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The Formation of L-Glutamic Acid from α-Ketoglutaric Acid by a Constructed Enzyme System

By Kenji TAKAHASHI* and Yoichi KONDO**

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Received August 11, 1958

The L-glutamic acid dehydrogenase reaction was successfully coupled with the hydrogenase reaction to use gaseous hydrogen for the reduction of α-ketoglutaric acid to L-glutamic acid. The condition and the stoichiometry of the reaction were investigated.

INTRODUCTION

It is a well-known and biochemically important reaction[1] that α-ketoglutaric acid is reduced to L-glutamic acid in the presence of L-glutamic acid dehydrogenase, reduced DPN or TPN and ammonium ion.

The present paper deals with the coupling of this reaction with the hydrogen-hydrogenase system which supplies reduced DPN continuously. The use of hydrogen as a reducing agent for this reaction is of interest especially, for industrial purposes.

The reaction is schematically shown as follows:-(Fig. 1)

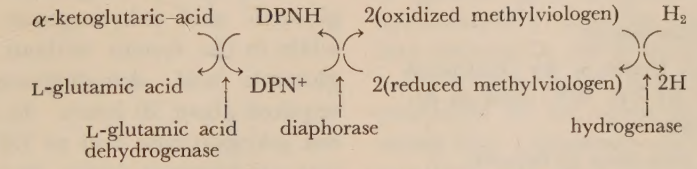

FIG. 1. The Constructed Enzyme System

MATERIALS AND METHODS

Materials

L-glutamic acid dehydrogenase was extracted from beef liver and purified as described by Iwatsubo et al.[2]

Diaphorase was extracted from pig heart and purified by the method of Straub[3].

As the hydrogenase source were employed the cell-free extracts of *Desulfovibrio desulfuricans* obtained by ultrasonic disintegration[4].

DPN was extracted from baker's yeast and purified by the method of Williamson and Green[5].

Methods

The hydrogen uptake was measured by Warburg's apparatus. The contents of the Warburg's vessel were as follows. The main chamber contained 0.1 ml of the L-glutamic acid dehydrogenase preparation (30 mg protein per ml), 0.05 ml of the diaphorase preparation (2.5 mg protein per ml), 0.05 ml of 8×10^{-4}M DPN, 0.2 ml of 0.01 M methylviologen, 0.1 ml of 0.114M α-ketoglutaric acid, 0.1 ml of 0.24M ammonium chloride and 0.4 ml of 0.1M phosphate buffer, pH 7.3. The side chamber contained 0.3 ml of the hydrogenase preparation (23 mg protein per ml). The center well was occupied with 0.2 ml of 40% potassium hydroxide aq. solution. The total volume was 1.5 ml. The reaction

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1) J.A. Olson, *Fed. Proc.*, **11**, 266 (1952).
2) M. Iwatsubo, H, Watari, T. Soyama, K. Ito, M. Nishimiki, and K. Hiraoka, Proceedings of the 9th symposium on enzyme chemistry (Japan), p. 36.
3) E. B. Straub, *Biochem. J.*, **38**, 787 (1939).
4) Y. Kondo, T. Kameyama, and N. Tamiya, *J. Biochem.*, **44**, 61 (1957).
5) S. Williamson, and D. E. Green, *J. Biol. Chem.*, **135**. 345 (1940).

was carried out at pH 7.0 at 30°C under an atmosphere of hydrogen.

The L-glutamic acid content was measured enzymatically according to the method of Gale et al.[6]. The dinitrophenylation method was also used for the same purpose[7].

RESULTS AND DISCUSSION

Factors required for the reaction

The results of an experiment are shown in Fig. 2.

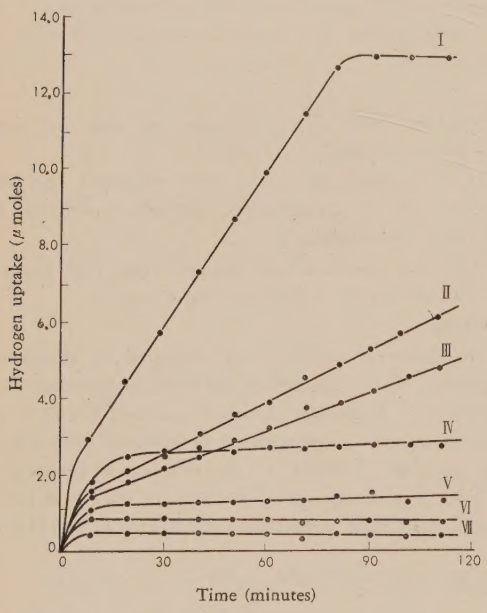

FIG. 2. Hydrogen Uptake in the Constructed Enzyme Systems at pH 7.0, 30°C under an Atmosphere of Hydrogen.

The numbers in the figure denote the following: I, Complete system containing 0.1 ml of L-glutamic acid dehydrogenase preparation (30 mg protein per ml), 0.05ml of diaphorase preparation (2.5 mg protein per ml), 0.3 ml of the hydrogenase preparation (23 mg protein per ml), 0.05 ml of 8×10^{-4}M DPN, 0.2 ml of 0.01M methylviologen, 0.1 ml of 0.114M α-ketoglutaric acid, 0.1 ml of 0.24M NH_4Cl and 0.4 ml of 0.1M phosphate buffer, pH 7.3. II, System without diaphorase; III, without L-glutamic acid dehydrogenase; IV, without NH_4Cl; V, without DPN; VI, without α-ketoglutaric acid; VII, without methylviologen.

In the complete system (curve I) which contained hydrogen, hydrogenase, methylviologen, diaphorase, DPN, L-glutamic acid dehydrogenase, α-ketoglutaric acid and ammonium ion, the uptake of hydrogen lasted for 85 minutes and amounted to 12.2 μ moles. The system without α-ketoglutaric acid (curve VI) was taken as the control. This amount was in good agreement with that of α-ketoglutaric acid added (11.4 μ moles). The uptake of hydrogen was also observed in the system without diaphorase (curve II) or L-glutamic acid dehydrogenase (curve III), but its velocity was about one third of that of the complete system. This may be due to the insufficient purification of the enzymes.

S. Korkes et al[8]. reported that the hydrogenase from *Cl. kluyveri* reduced DPN directy. The results shown above, however, indicate the requirement for methylviologen and diaphorase, suggesting that the hydrogenase from *Desulfovibrio desulfuricans* does not reduce DPN directly. The results obtained in the manometric experiments mentioned above coincided with those obtained in the preliminary experiments where DPNH was supplied continuously by the reduction of DPN with the substrate amounts of reduced methylviologen. Reduced methylviologen of blue color was oxidized and decolorized in 27 minutes in the complete system consisting of α-ketoglutaric acid, DPN, L-glutamic acid dehydrogenase and diaphorase, while in the system without diaphorase or L-glutamic acid dehydrogenase decolorization required about 20 hours. In the system without α-ketoglutaric acid or DPN, decolorization was not observed within 30 hours.

The hydrogen uptake of 2 μ moles was observed in the system that was not added with ammonium ion (curve IV), which may be ascribed to the fact that the ammonium ion was not completely eliminated from the hydrogenase preparation by dialysis.

In the system without DPN (curve V), the uptake of hydrogen was hardly detectable.

The L-glutamic acid produced was determined

6) E. F. Gale, *Biochem. J.*, **39**, 46 (1945), **41**, vii (1947).
7) F. Sanger, *Biochem. J.*, **39**, 507 (1945).
8) S. Korkes, *J. Biol. Chem.*, **216**, 737 (1945).

by the dinitrophenylation method. It was 7.6 μ moles in the complete system, which was 62 per cent of the hydrogen consumed, and almost none in the systems V, VI, and VII. In the systems II and III, the semiquantitative determination of L-glutamic acid formed gave the values corresponding to about two-thirds of the hydrogen uptake.

From the results given above, it is concluded that all the factors involved in the complete system are essential for the reaction intended.

In these experiments, hydrogen was utilized to reduce DPN through hydrogen-hydrogenase-methylviologen-diaphorase system. Methylviologen, being an artificial electron carrier, some intermediary carrier might be present in *Desulfovibrio desulfuricans* having hydrogenase. Cytochrome c_3[9,10,11] of *D. desulfuricans*, however, could not be substituted for methylviologen.

The reaction might be of industrial use as a method for the reduction of organic compounds such as α-ketoglutaric acid in the presence of dehydrogenases specific to them.

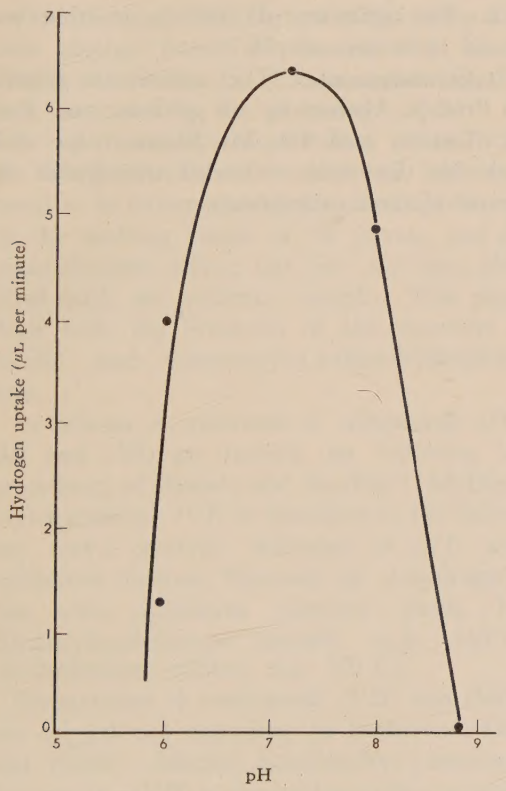

FIG. 3. The Velocities of Hydrogen Uptake at Various pH.

The reaction conditions were the same with the complete system (I, in Fig. 2) except pH.

Conditions of the reaction

Comparing the reaction velocities at various pH values, the optimum pH for this enzyme system was found to lie around pH 7.

Stoichiometry of the reaction

The reaction was carried out with varying amounts of α-ketoglutaric acid under similar conditions as mentioned above.

The correlation between the amount of hydrogen uptake and that of L-glutamic acid production was examined (Table I). The latter was determined enzymatically.

In every case, the amount of hydrogen uptake agreed well with that of α-ketoglutaric acid added almost quantitatively within the limit of experimental errors. This reaction may therefore be utilized for the determination of α-ketoglutaric acid.

On the other hand, the L-glutamic acid formation was determined to be 66 per cent on the basis of hydrogen uptake on the average, approximately coinciding with the value of 62 per cent obtained by the dinitrophenylation method.

From these results, it may be assumed that two-thirds of the α-ketoglutaric acid is converted into L-glutamic acid and the rest, the one-third, into other products.

The possibility that L-glutamic acid produced was subjected to further transformation into other substances was denied by the fact that 100 per cent of L-glutamic acid was recovered in a system to which L-glutamic acid was added instead of α-ketoglutaric acid. (See system V in Table I.)

9) M. Ishimoto, and J. Koyama, *Bull. Chem. Soc. Japan*, **28**, 232 (1955).
10) M. Ishimoto, J. Koyama, T. Yagi, and M. Shiraki, *J. Biochem.*, **44**, 413 (1957).
11) J. Postgate, *Biochem. J.*, **56**, xi (1954).

TABLE I. STOICHIOMETRY OF THE REACTION

System	α-Ketoglutaric acid added (μ moles)	Hydrogen uptake (μ moles)	L-Glutamic acid formation (μ moles)	Molar ratio of Hydrogen uptake to α-Ketoglutaric acid added (%)	Molar ratio of L-Glutamic acid formed to Hydrogen uptake (%)
I	11.4	11.0	7.8	97	71
II	5.7	5.8	3.2	100	57
III	2.9	2.7	1.8	94	69
IV	0	0	0	0	–
V	0 (L-Glutamic acid 10.0 μ moles)	–	10.0	–	–

SUMMARY

1. The L-glutamic acid dehydrogenase reaction was successfully coupled with hydrogenase reaction utilizing gaseous hydrogen for the reduction of α-ketoglutaric acid.

The system consisted of hydrogen, hydrogenase, methylviologen, diaphorase, DPN, L-glutamic acid dehydrogenase, α-ketoglutaric acid and ammonium ion.

2. The amount of hydrogen uptake agreed well with the amount of α-ketoglutaric acid added.

The amount of L-glutamic acid produced was determined to be about 66 per cent of the hydrogen uptake.

3. The optimum pH for the reaction was found to be around pH 7.

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Terpenoids. Part V.
The Synthesis of Occidol

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Received September 3, 1958

The structure (I) of occidol, a new sesquiterpene alcohol isolated from the essential oil of the wood of *Thuja occidentalis* L. has been proposed in a recent paper. The structure is confirmed by (a) comparison of the infrared spectrum of the synthesized racemate with that of occidol, and (b), by the mixed m.p. of picrate of the synthesized 6-isopropyl-1,4-dimethylnaphthalene with that of a dehydrogenated product of occidol.

Occidol, m.p. 69-70°C, is a tertiary sesquiterpene alcohol found in the essential oil of *Thuja occidentalis* L. The structure (I) has been proposed in the preceding paper[1]. In that report, an alkyl naphthalene obtained by the dehydrogenation of occidol has been supposed to be 6-isopropyl-1,4-dimethylnaphthalene by the melting points of its picrate and the trinitrobenzene adduct but has not been identified with an authentic sample. This paper deals with the syntheses of the racemate of occidol and 6-isopropyl-1,4-dimethylnaphthalene.

In Scheme A, syntheses of compounds (IV), (V) and (VI) are carried out following the procedures of Barnett and Sanders.[2] 5,8-Dimethyl-1-tetralone (VI) is identified in the following way: catalytic reduction of (VI) with palladium charcoal, followed by dehydrogenation with palladium charcoal yields 1,4-dimethylnaphthalene (picrate, m.p. 140°C; trinitrobenzene adduct, m.p. 160°C).

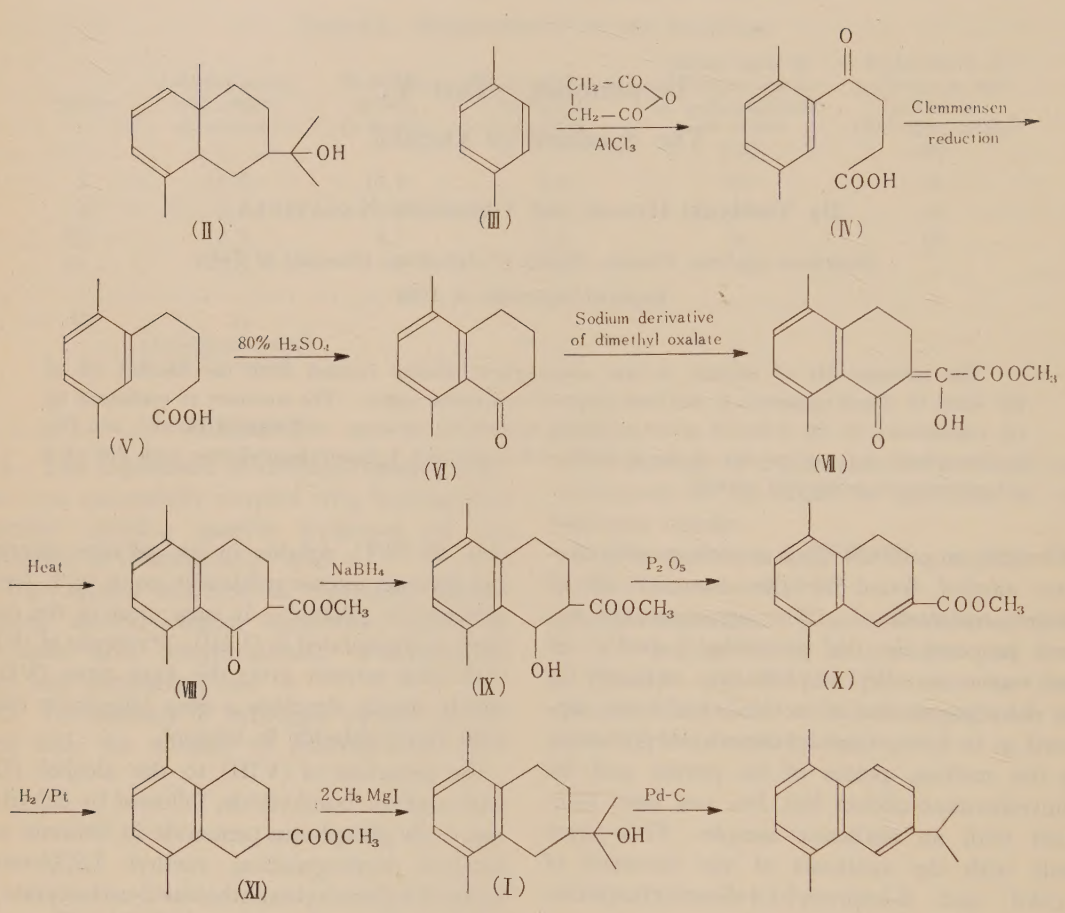
Preparations of compounds (VII) and (VIII) are carried out according to Bachmann, Cole and Wilds[3]. Methyl 5,8-dimethyl-1-tetralone-2-glyoxalate (VII) is obtained as yellow crystals, m.p. 69–70°C, soluble in an alkaline solution and gives an intense red-brown color with ferric chloride in ethanol. It may exist in the enol form as formulated in (VIII). Pyrolysis of (VII) with glass powder gives the keto ester (VIII), which slowly develops a deep blue-green color with ferric chloride in ethanol.

On reduction of (VIII) to the alcohol (IX) with sodium borohydride, followed by dehydration with phosphorus pentoxide in benzene and catalytic hydrogenation, methyl 1,2,3,4-tetrahydro-5,8-dimethylnaphthalene-2-carboxylate (XI) is obtained in a good yield. The compound (XI) is reacted with methyl magnesium iodide in the usual manner. The product (I) is obtained as colorless crystals, m.p. 101-102°C, having an infrared absorption spectrum identical with that of natural occidol (Figs. 1 and 2).

Furthermore, the dehydrogenation of the compound (I) with 5% palladium charcoal gives 6-isopropyl-1,4-dimethylnaphthalene, the picrate of which melts at 102-103°C, undepressed by that prepared from natural occidol.

The structure of occidol has thus been definitely established. It is of interest to note that an alcohol, containing an aromatic nucleous in the molecule which is closely related to eudalene, has been isolated for the first time.

1) Y. Hirose and T. Nakatsuka, This Bulletin, **23**, 143 (1959).
2) E. De Barry Barnett and F.G. Sanders, *J. Chem. Soc.*, **1933**, 434.
3) W.E. Bachmann, W. Cole and A. L. Wilds, *J. Am. Chem. Soc.*, **62**, 824 (1940).



Scheme A

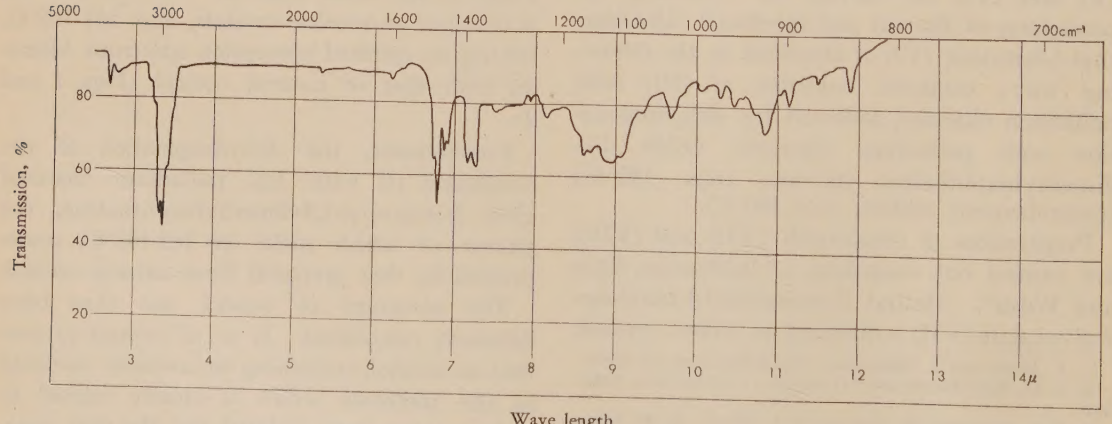


FIG. 1. Natural *d*-Occidol (in CCl_4, 0.1 mm)

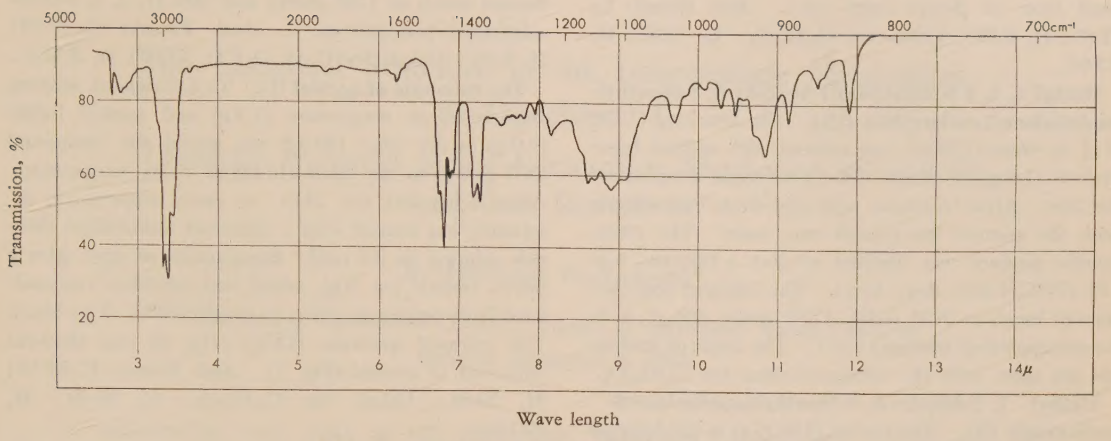


FIG. 2. Synthesized *dl*-Occidol (in CCl_4, 0.1 mm)

We have already isolated occidentalol (II) of eudalene type from the same essential oil[4]. The fact that occidol is accompanied by occidentalol seems to suggest that the latter would be biogenetically transformed to the former by rearrangement involving the migration of an angular methyl group.

EXPERIMENTAL

3-(2, 5-Dimethylbenzoyl)-propionic acid (IV) Powdered aluminium chloride (300 g) was slowly added to a mixture of *p*-xylene, b.p. 135.5–136.0°C (120 g), succinic anhydride (100 g) and tetrachloroethane (350 cc). The reaction mixture was kept overnight and treated with ice and hydrochloric acid. The product, b.p. 186–189°C/2 mm, m.p. 80°C (110 g; 47%) was obtained, (lit[2]. m.p. 86°C).

4-(2, 5-Dimethylphenyl)-butyric acid (V). A solution of the above acid (IV) (67 g) in toluene (300 cc) was mixed with amalgamated zinc (120 g), concentrated hydrochloric acid (200 cc) and water (75 cc) and the mixture was refluxed on a sand bath for 30 hr. Evaporation of toluene under reduced pressure gave a crystalline mass, recrystallized from petroleum ether, (50g; 81%). It did not show a sharp melting point, ca. 60°C, (lit[2]. 70°C).

5, 8-Dimethyl-1-tetralone (VI). The acid (V) (75 g) was heated with 80% sulfuric acid (400 cc) on a water bath for 5 hr. The ether-soluble product was distilled to give a fraction, b.p. 134–135°C/6 mm, (41 g; 60%). λ_{max} 255 and 305 mμ (log ε 4.0 and 3.3, respectively), infrared maximum at 1678 (Ph–CO–) cm^{-1}.

1, 4-Dimethylnaphthalene. 5, 8-Dimethyl-1-tetralone (500 mg), palladium charcoal (50 mg) and glacial acetic acid (30 cc) were shaken together in hydrogen until absorption was ceased (30 hr.). Filtration and evaporation in vacuo gave an oil (500 mg). This liquid was heated with 5% palladium charcoal (100 mg) under reflux for 5 hr. The petroleum-ether-soluble product gave a picrate, m.p. 139–140°C and a trinitrobenzene adduct, m.p. 159–160°C. *Anal.* of picrate. Found: N, 11.07. Calcd. for $C_{18}H_{15}O_7N_3$: N, 10.91%.

Methyl 5, 8-dimethyl-1-tetralone-2-glyoxalate (VII). According to the procedure of Bachmann, Cole and Wilds[3], to a solution of the sodium derivative of dimethyl oxalate in dry benzene (500 cc), which was prepared from sodium (2.0 g), absolute methanol (30 cc) and dimethyl oxalate (11 g), was added a solution of 5, 8-dimethyl-1-tetralone (VI) (9 g) in dry benzene (60 cc) in an atmosphere of dry nitrogen. After the mixture was swirled at room temperature for 5 hr., the benzene solution was extracted with 2% sodium hydroxide solution. Acidification of the alkaline solution gave yellow crystalline glyoxalate, m.p. 69–70°C (from acetone), (12.5 g; 93%). *Anal.* Found: C, 70.08; H. 6.42. Calcd. for $C_{15}H_{16}O_4$: C, 69.21; H, 6.20%.

Methyl 5, 8-dimethyl-1-tetralone-2-carboxylate (VIII). The above-glyoxalate (12 g) was heated with powdered soft glass (6 g) at 180°C. After a vigorous evolution of carbon monoxide, the ether-soluble product was distilled to give a fraction, b.p. 148–152°C/4 mm, (8.5 g; 79%). The fraction was crystallized from ethanol, m.p. 75–76°C. The infrared spectrum (Nujol) showed bands at 1733 (ester), 1672 (Ph-CO-) and 1650

4) T. Nakatsuka and Y. Hirose, This Bulletin, **20**, 215 (1956).

(enol form of β-keto ester) cm^{-1}. *Anal.* Found: C, 72.69; H, 6.92. Calcd. for $C_{14}H_{16}O_3$: C, 72.39; H, 6.94%.

Methyl 1, 2, 3 4-tetrahydro-1-hydroxy-5, 8-dimethylnaphthalene-2-carboxylate (IX). The keto ester (VIII) (7 g) in ethanol (30 cc) was reduced with sodium borohydride (1.5 g) in ethanol (50 cc) at room temperature for 5 hr. After treatment with cold dilute hydrochloric acid, the mixture was poured into water. The ether-soluble product was distilled to give a fraction, b.p. 170–174°C/4 mm, (6 g; 85%). The infrared spectrum showed bands at 3425 (OH), 1718 (ester), 806 (1, 2, 3, 4-tetrasubstituted benzene) cm^{-1}. The result of analysis did not agree with the calculated value for $C_{14}H_{18}O_3$.

Methyl 1, 2-dihydro-5, 8-dimethylnaphthalene-3-carboxylate (X). The alcohol (IX) (6 g) in dry benzene (30 cc) was added to a suspension of phosphorus pentoxide (2.6 g) in dry benzene (10 cc) with stirring in the cold. The mixture was poured into water and extracted with ether. The ether-soluble product was distilled to give a fraction, b.p. 145–150°C/4 mm, (3.5 g; 64%). The infrared spectrum showed bands at 1698 ($\alpha\beta$-unsaturated ester), 1629 (C=C conjugated with benzene) and 806 (1, 2, 3, 4-tetrasubstituted benzene) cm^{-1}. *Anal.* Found: C, 77.22; H, 8.29. Calcd. for $C_{14}H_{16}O_2$: C, 77.75; H, 7.46%.

Methyl 1, 2, 3, 4-tetrahydro-5, 8-dimethylnaphthalene-2-carboxylate (XI). The above unsaturated ester (X) (3.5 g) in ethanolacetic acid (2: 1) was hydrogenated using Adams' catalyst until absorption was ceased (17 hr.). After working up the reaction mixture in the usual manner, the product (3.1 g; 90%), b.p. 142–145°C/2 mm, was obtained. The infrared spectrum showed bands at 1739 (ester) and 806 (1, 2, 3, 4-tetrasubstituted benzene) cm^{-1}. *Anal.* Found: C, 79.10; H, 8.79. Calcd. for $C_{14}H_{18}O_2$: C, 77.03; H, 8.31%.

The racemate of occidol (I). To a Grignard solution prepared from magnesium (1.2 g) and methyl iodide (9.0 g) in dry ether (30 cc) was added the compound (XI) (3.0 g) in dry ether (15 cc) at room temperature. After being left for 24 hr. at room temperature, the mixture was treated with a saturated ammonium chloride solution in the cold. Evaporation of ether gave a brown residue (ca. 3 g), which was solidified, recrystallized from petroleum ether, m.p. 101-102°C, (1 g; 33%). The infrared spectrum (CCl_4) (Fig. 2) was identical with that of occidol (Fig. 1). *Anal.* Found: C, 82.54; H, 10.61. Calcd. for $C_{15}H_{22}O$: C, 82.51; H, 10.16%.

6-Isopropyl-1, 4-dimethylnaphthalene (XII). The racemate of occidol (100 mg) was dehydrogenated with 5% palladium charcoal (100 mg) for 2 hr. The product was passed through a column of alumina and eluted with petroleum ether to afford a liquid, which gave picrate, m.p. 102–103°C, undepressed by that prepared from natural occidol. *Anal.* Found: N, 9.87. Calcd. For $C_{21}H_{21}O_7N_3$: N, 9.8%.

Acknowledgements. The authors are indebted to Mr. K. Aizawa, the Faculty of Agriculture, University of Tokyo, for infrared analyses. Thanks are due to the Faculty of Agriculture, University of Tokyo for performing the microanalyses. This experiment was supported in part by a grant from the Ministry of Education.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 4, p. 257~259, 1959]

The Chemical Structure of the Intermediate Metabolites of Catechin. I

Chemical Properties of the Intermediate Metabolites (G and H) and their Derivatives

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Received September 5, 1958

Examinations were made on two intermediate metabolites excreted after administration of (+)-catechin to the rabbit. From the infrared and ultraviolet spectra of these two substances ($C_{11}H_{12}O_3$ and $C_{11}H_{12}O_4$) and chemical properties of their various derivatives, they were identified as 3-hydroxyphenyl lactone and 3, 4-dihydroxyphenyl lactone. However, it still remains unknown whether they are δ- or γ-lactone.

It has previously been reported[1] that, from the urine of rabbits administered orally with (+)-catechin, a substance of m.p. 105-106°, $C_{11}H_{12}O_3$ (G), and a substance of m.p. 145-146°, $C_{11}H_{12}O_4$ (H), were isolated from the neutral portion and both were assumed to be hydroxyphenyl lactones from their color reactions and other properties. It was considered necessary to establish the correct chemical structure of these substances in order to elucidate the metabolic mechanism of catechin. Therefore, infrared and ultraviolet spectra of these substances were measured, and various derivatives were prepared, of which examinations offered a certain amount of evidences for the decision of the chemical structure of these intermediate metabolites.

Infrared absorption spectra indicated the similarity in chemical structure of these two substances and showed the presence of hydroxyl and lactone in both. Characteristic absorptions due to out-of-plane vibration of C-H in the benzene ring appeared at 12.60, 13.33, and 14.35μ in G, which suggested 1,3-substitutions, and at 11.85, 12.25, and 12.45 μ in H, suggesting 1,3,4-substitutions.

From the results of color reactions, it has earlier been shown that G possesses one phenolic hydroxyl and H two phenolic hydroxyls in a vicinal position. Considering the result of infrared spectral analysis, G was assumed to be the 3-hydroxyphenyl type and H, the 3,4-dihydroxyphenyl type. Comparison of the ultraviolet spectrum of G with that of *m*-cresol and of 3,4-dihydroxy-ethylbenzene showed, as will be seen from Figs. 2 and 3, that their absorption curves are entirely identical, with good agreement of absorption maxima.

The methyl derivatives obtained by methylation of the phenolic hydroxyl with diazomethane were as the result of analytical values found to be a monomethyl derivative from G and a dimethyl derivative from H, and their elemental analytical values agreed well with those calculated. Infrared absorption spectra of these derivatives are shown in Fig. 4 from which it is seen that the absorption of hydroxyl in Fig. 1 has disappeared completely and that there is no other hydroxyl in the molecule. In other words, substance G contains one

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1) Y. Oshima and H. Watanabe, *J. Biochem.* (*Japan*), **45**, 973 (1958).

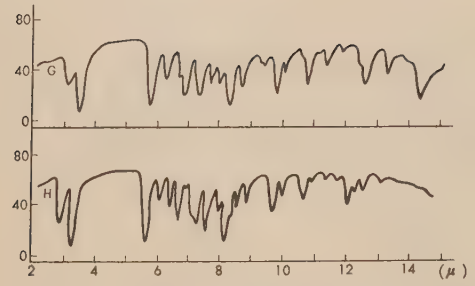

FIG. 1. Infrared Absorption Spectra of G and H (Nujol, NaCl prism)

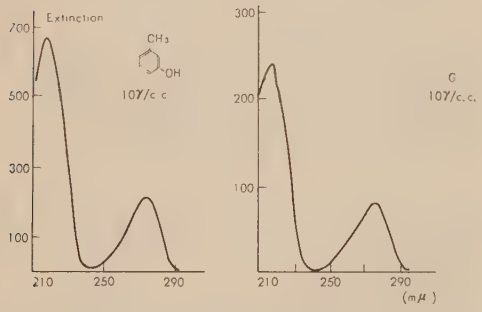

FIG. 2. Ultraviolet Absorption Spectra of G and *m*-Cresol (in EtOH)

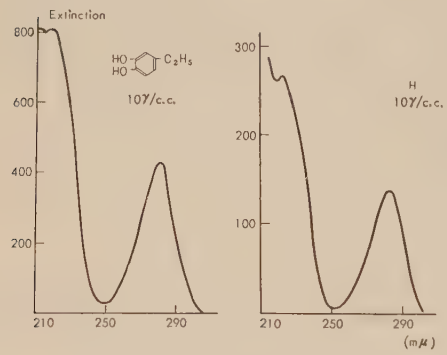

FIG. 3. Ultraviolet Absorption Spectra of H and 3,4-Dihydroxyethylbenzene (in EtOH)

phenolic hydroxyl and substance H has two phenolic hydroxyls.

Benzoylation with *p*-nitrobenzoyl chloride in pyridine afforded monobenzoyl derivative from G and dibenzoyl derivative from H, their elemental analytical values agreeing well with those calculated, and the number of hydroxyl

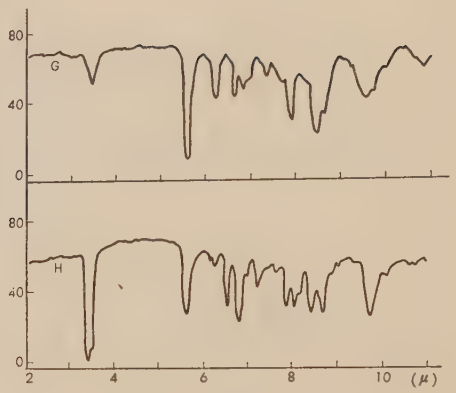

FIG. 4. Infrared Absorption Spectra of Methyl Derivatives of G (in Liquid, NaCl prism) and H (Nujol, NaCl prism)

groups were in accord with those observed in the foregoing methyl and dimethyl derivatives. This has entirely ruled out the possibility of the presence of other phenolic hydroxyls and alcoholic hydroxyls.

A lactone type was assumed for the carbonyl and cleavage of the lactone ring by heating with alcohol saturated with ammonia afforded an acid amide whose analytical values were in good agreement with the theoretical. Infrared spectra of the acid amides, as shown in Fig. 5, indicated complete disappearance of carbonyl absorptions at 5.75 μ in G and at 5.76 μ in H, and the appearance of a characteristic absorption for acid amide at 5.55 μ in G and 5.65 μ in H. These evidences show that the original substances are lactone compounds.

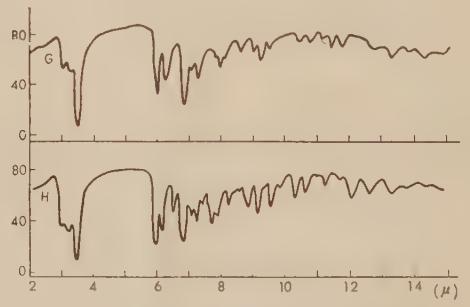

FIG. 5. Infrared Absorption Spectra of Acid Amide Derivatives of G and H (Nujol, NaCl prism)

Bromination of H in chloroform afforded a tribromo derivative whose analytical values were in good agreement with calculated values.

Considering the results of infrared and ultraviolet spectral meassurements, as well as from various properties of derivatives, G is considered to be 3-hydroxyphenyl lactone and H, 3,4-dihydroxyphenyl lactone, but the nature of the lactone rings in both yet remained undetermined. Infrared spectral analysis of G and H indicated the carbonyl to be in the position for absorption of a six-membered ring while the spectra of their methyl derivatives indicated the absorption of a five-membered ring. The skeletal structures will probably be revealed by the following oxidative decomposition and syntheses.

EXPERIMENTAL

Methyl derivative of G: To a solution of 100 mg of G in 0.5 ml of ethanol, 20 ml of ethereal diazomethane (prepared from 0.5 g of nitroso N-methyl urethane) was added, and it was allowed to stand for three days in an ice box and then for one day at room temperature. After evaporating the solvents and diazomethane the residue was dissolved in 20 ml of ether. The ether solution was shaken with one per cent sodium hydroxide solution, washed with water, dried, evaporated and distilled under reduced pressure giving 90 mg of colorless liquid; b.p. 0.8, 148–150°. *Anal.* Found: C, 70.00; H, 6.67. Calcd. for $C_{12}H_{14}O_3$: C, 69.88; H, 6.84.

Methyl derivative of H: Two hundred milligram of H was added to the solution of diazomethane in 20 ml of ether (prepared from 0.5 g of nitroso N-methyl urethane) and it was allowed to stand in an ice box for four days. The ether solution was concentrated to about 5 ml and after being allowed to stand one day, the crystalline methyl derivative was collected and recrystallized from a mixture of mathanol and ether, colorless prisms; m.p. 87–88°. 130 mg. *Anal.* Found: C, 66.12; H, 6.65. Calcd. for $C_{13}H_{16}O_4$: C, 66.08; H, 6.83.

***p*-Nitrobenzoyl derivative of G:** To a solution of 25 mg of H in 0.5 ml of absolute pyridine 50 mg of *p*-nitrobenzoyl chloride in 0.5 ml of absolute pyridine was added under cooling. After standing for twenty-four hours at room temperature 10 ml of ice water was added and crystals were collected, washed with a diluted ammonium hydroxide solution and recrystallized from ethanol in slight yellow needles; m.p. 78°. 15 mg. *Anal.* Found: C, 63.45; H, 4.65; N, 4.37. Calcd. for $C_{18}H_{15}O_6N$: C, 63.34; H, 4.43; N, 4.15.

***p*-Nitrobenzoyl derivative of H:** In this experiment the same procedure as H was carried out and 40 mg of *p*-nitrobenzoate was obtained from 25 mg of H and 100 mg of *p*-nitrobenzoyl chloride. Recrystallization from ethanol gave scales; m.p. 139–140°. 20 mg. *Anal.* Found: C, 59.43; H, 3.64; N, 5.34. Calcd. for $C_{25}H_{18}O_{10}N_2$: C, 59.28; H, 3.56; N, 5.53.

Acid amide derivative of G: A solution of 100 mg of G in 6 ml of ammonia saturated absolute ethanol was heated for ten hours at 100° in a sealed glass tube. After cooling, ethanol and ammonia were evaporated and recrystallized from a mixed solution of ethanol and benzol. Colorless scales; m.p. 111°. *Anal.* Found: C, 63.41; H, 7.04; N, 6.61. Calcd. for $C_{11}H_{15}O_3N$: C, 63.14; H, 7.22; N, 6.69.

Acid amide derivative of H: The method employed in this experiment was the same as descrived in the above H, using 60 mg of H and 4 ml of ammonia saturated absolute ethanol. Recrystallization from ethanol-benzol gave slight yellow scales; m.p. 129–130°. *Anal.* Found: C, 59.39; H, 6.92; N, 6.12. Calcd. for $C_{11}H_{16}O_4N$: C, 58.65; H, 6.71; N, 6.21.

Bromo derivative of H: A suspension of 30 mg of H in 4 ml of absolute chloroform was cooled in ice water and a chloroform solution of bromine (5 per cent) was added dropwise, until absorption of bromine was completed.

The reaction mixture was allowed to stand at room temperature and the crystals were collected, washed with chloroform and recrystallized from acetic acid. Colorless needless; m.p. 208–209°. *Anal.* Found: C, 30.09; H, 2.07. Calcd. for $C_{11}H_9O_4Br_3$: C, 29.66; H, 2.02.

The author expresses his deep gratitude to Prof. Y. Oshima for his helpful guidance throughout this work, and to Dr. M. Fujisawa and Dr. N. Sugimoto of the Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd., for their kind encouragement. He is also indebted to Mr. K. Kodera of the Osaka Research Laboratory for infrared spectral data and to Mr. M. Shido of the elemental analysis service center of Kyushu University for microanalyses.

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The Chemical Structure of the Intermediate Metabolites of Catechin. II

Oxidative Decomposition of the Intermediate Metabolites (G and H)

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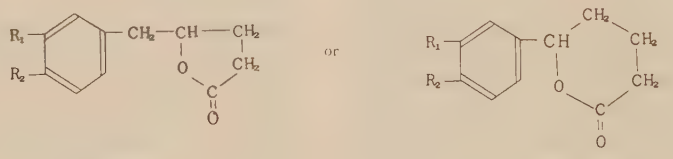
Received September 5, 1958

In order to confirm the chemical structures of 3-hydroxyphenyl lactone and 3, 4-dihydroxyphenyl lactone assumed for the intermediate metabolites G and H, respectively, oxidation of the methyl derivatives of G and H with potassium permanganate was carried out. The products of oxidation obtained from G were 3-methoxybenzoic, succinic, and malonic acids, which indicated the structure of G to be δ-(3-hydroxyphenyl)-γ-valerolactone, and further the structure of H was similarly revealed to be δ-(3, 4-dihydroxyphenyl)-γ-valerolactone.

It had been shown in the preceding report that chemical structures of the intermediate metabolites of (+)-catechin were assumed as 3-hydroxyphenyl lactone for G and 3,4-dihydroxyphenyl lactone for H, as a results of infrared and ultraviolet spectral measurements and from properties of their various derivatives. As for the details of the structure, especially the nature of the lactone ring, γ-lactone seems to be most probable considering the structure of catechin, (catechin has a hydroxyl group in 3-position) and the position of carbonyl absorption in the infrared spectra of the methyl derivatives of G and H. However, substances such as G and H, per' se', from infrared spectral meaurements failed to be proved as γ- or δ-lactone. Thus, the two probable structure for G and H would be as follows:



G: R_1=OH, R_2=H　　H: R_1=R_2=OH

As it was thereby considered that the oxidative decomposition and confirmation of the acids formed would reveal the nature of the lactone ring and establish the substituted position on the benzene ring, the oxidation of methyl derivatives of G and H with potassium permanganate was therefore carried out. The main objective of the oxidation was to sever the lactone at the alcoholic hydroxyl so that alkaline oxidation should be carried out after cleavage of the lactone ring with dilute alkali.

Permanganate oxidation of the methyl derivative of G afforded 3-methoxybenzoic acid and that of the methyl derivative of H gave 3,4-dimethoxybenzoic acid, both in the crystalline form. Other decomposition products were either too small in quantity or not isolated in crystalline form. Therefore, the filtrate obtained after

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separation of these acids was extracted with ether to obtain water-soluble acids, which were led to hydroxamic acid derivatives according to the method of Inoue and Noda[1] and submitted to paper partition chromatography[2].

The chromatogram of the methyl derivative of G (Table I and Fig. 1) indicated seven spots (a, b, c, d, e, f, g) whose R_F values suggested the formation of succinic and malonic acids. Comparison of R_F values of hydroxamic acid derivatives with pure samples of these acids revealed that spots a, c, e, and g were identical with corresponding derivatives of succinic acid, and spots b, d, and e with those of malonic acid. Spot f was found to be the hydroxamic acid derivative of 3-methoxybenzoic acid.

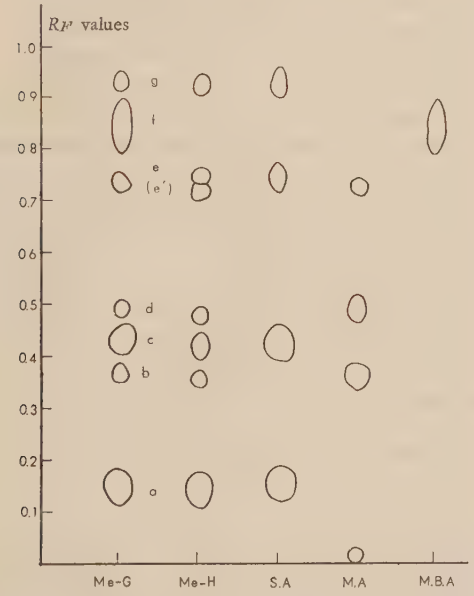


FIG. 1. Paper Partition Chromatogram of Hydroxamic Acid Derivatives of Oxidation Products (Water-soluble Acids) of Methyl Derivatives of G and H, and of Succinic, Malonic, and 3-Methoxybenzoic Acid.

Me-G: Methyl derivative of G
Me-H: Methyl derivative of H
S. A. : succinic acid
M. A.: malonic acid
M.B.A.: 3-methoxybenzoic acid

1) Y. Inoue and M. Noda, *J. Agr. Chem. Soc. Japan*, **24**, 291 (1951).
2) Y. Inoue and M. Noda, *J. Agr. Chem. Soc. Japan*, **24**, 295 (1951).

Methyl derivatives of H showed six spots other than f which were identified similarly with succinic and malonic acids. The presence of 3,4-dimethoxybenzoic acid was not observed this probably being due to the fact that this acid is insoluble in water. Since malonic acid was detected, 3-methoxy- and 3,4-dimethoxyphenylacetic acids should have been present, but these acids were not detected either in crystalline state or in the chromatogram. From the size of the spot of malonic acid, the amount of this acid formed in the metabolite seems to have been smaller than that of succinic acid and consequently, the amount of these phenylacetic acids would be small and the majority of these acids must have undergone secondary decomposition, this, probably being the reason why they were not detected.

TABLE I. R_F VALUES OF PAPER CHROMATOGRAM SHOWN IN FIG. 1.

Spots	Me-G	Me-H	Succinic acid	Malonic acid	3-Methoxy Benzoic acid
				0.02	
a	0.16	0.15	0.16		
b	0.37	0.36		0.36	
c	0.43	0.42	0.43		
d	0.49	0.48		0.49	
e (e')	0.74	0.72 0.74	0.75	0.73	
f	0.84				0.84
g	0.93	0.93	0.93		

Me-G: methyl derivative of G
Me-H: methyl derivative of H

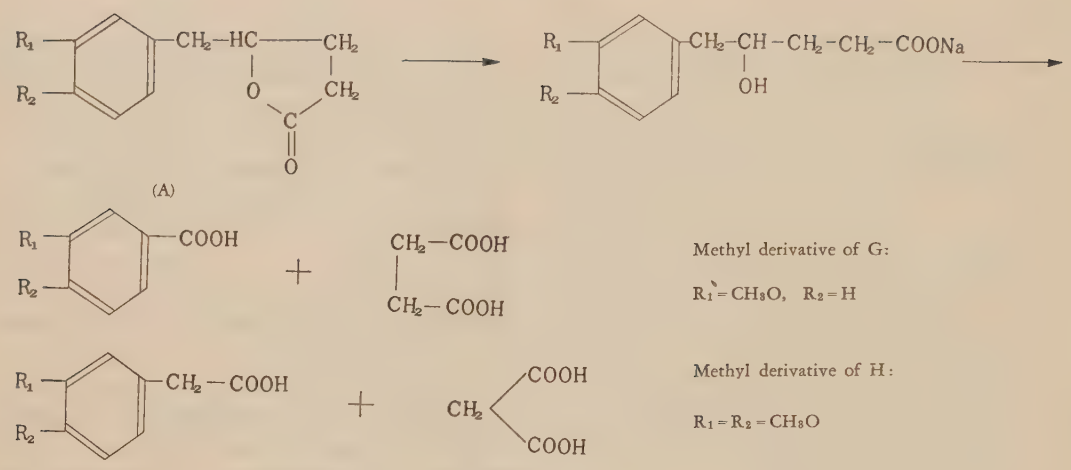
In any of these cases, glutaric acid derivatives were not detected.

Experimental results described in this paper and the preceding paper can satisfactorily be elucidated by application of the γ-lactone structure (A), to the methyl derivatives of G and H, as shown in Schema 1:

The structures of G and H were therefore assumed to be δ-(3-hydroxyphenyl)-γ-valerolactone and δ-(3,4-dihydroxyphenyl)-γ-valerolactone.

EXPERIMENTAL

Oxidation of methyl derivatives of G and H. One hundred and thirty-eight milligram of methyl derivative of G was dissolved in 20 ml of 2 per cent sodium



Schema 1.

hydroxide solution by heating. To this solution, 367 mg of potassium permanganate in 40 ml of water was added drop by drop over a period of thirty minutes at 70° with stirring. By the same procedure, 87 mg of methyl derivative of H in 15 ml of 2 per cent sodium hydroxide solution was oxidized with 185.8 mg of potassium permanganate in 20 ml of water.

Isolation and identification of 3-methoxybenzoic and 3, 4-dimethoxybenzoic acid. After oxidation of the methyl derivative of G was completed manganese dioxide was filtered by suction and the filtrate was concentrated to a volume of 3 ml and acidified with hydrochloric acid. After it was allowed to stand at room temperature, needle crystalls separated. The acidic solution was neutrallized with concentrated ammonium carbonate solution and extracted with ether to remove the neutral substances. The aqueous layer was acidified with hydrochloric acid and needle crystals were separated. The separated crystals were collected and recrystallized from water. Twenty milligram of needle crystals, which melted at 106–107° were obtained. By the same procedure, 10 mg of needle crystalls, melting at 180–181° were obtained from the methyl derivative of H. No depressions of melting points were observed upon admixture with an authentic 3-methoxy benzoic acid and 3, 4-dimethoxy benzoic acid, respectively.

Extraction of water soluble acids. After removing 3-methoxybenzoic acid, the filtrate was saturated with sodium chloride and extracted with ether for twenty hours continuously. The ether solution was dried with anhydrous sodium sulfate and the ether was evaporated. In like manner the filtrate from which 3, 4-dimethoxybenzoic acid was removed, was extracted.

Preparation of ethyl hydroxamate. Each 10 mg of the above ether extracts were esterified with 4 ml of 95 per cent ethanol and 4 drops of concentrated hydyosulfuric acid under heating at 80–85° for thirty minutes. After cooling, the reaction mixtures were neutrallized with alcoholic potassium hydroxide solution. To these, 1.2 ml of alcoholic 5 per cent hydroxylamine hydrochloride and 1 ml of alcoholic 10 per cent potassium hydroxide solution were added and the mixtures maintained at 80–85° for ten minutes. The precipitates were filtered and filtrates were concentrated on a water bath until they were dried up, and after cooling the residues were neutrallized with alcoholic hydrochloric acid. The same procedure was carried out with succinic and malonic acid.

Paper partition chromatography of ethyl hydroxamate. Ethyl hydroxamates were subjected to paper partition chromatography using a filter paper of Toyo Roshi No. 50 and water saturated *n*-butanol as the solvent by a descending method. After development of 32–33 cm in length color was developed by spraying 10 per cent ferric chloride solution and R_F values were compared. The results are shown in Table 1 and Fig. 1.

The author expresses his deep gratitude to Prof. Y. Oshima for his helpful guidance throughout this work, and to Dr. M. Fujisawa and Dr. N. Sugimoto of the Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd., for their kind encouragement. He is also indebted to Mr. H. Shimamatsu for his technical assistance.

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The Chemical Structure of the Intermediate Metabolites of Catechin. III

Synthesis of the Intermediate Metabolites (G and H)

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Received September 5, 1958

Structures δ-(3-hydroxyphenyl)-γ-valerolactone and δ-(3, 4-dihydroxyphenyl)-γ-valerolactone were assigned respectively to the intermediate metabolites from (+)-catechin, G and H, according to experimental results reported in the preceding two papers. In the present series of work, *dl*-δ- (3-methoxyphenyl) and *dl*-δ-(3, 4-dimethoxyphenyl)-γ-valerolactones were synthesized and the infrared absorption spectra measured in carbon tetrachloride resulted to be entirely identical with the corresponding methyl derivatives of G and H. Racemic compounds of G and H were prepared by demethylation of these methyl derivatives.

dl-δ-(3,4-Diemthoxyphenyl)-γ-valerolactone has already been synthesized by Haworth in 1938[1], who recorded the melting point of this substance as 83-84°. The substance obtained in the present series of work by methylation of H with diazomethane melted at 87-88° and this difference was considered to be the difference between the optical isomers, dextrorotatory and racemic compounds, and not to be attributed to any structural difference. The synthesis of this compound was ascribed by Haworth to the following three routes: (Schema 1).

Of these three procedures, the liberation of the acetyl group in (2) and (3) is rather difficult and the yield is very poor. Consequently, procedure (1) seemed to be most suitable but it was considered possible that two kinds of addition reactions would occur in the reaction of 1,2-epoxy-3-(3,4-dimethoxyphenyl)-propane (I) and ethyl malonate, forming the following two addition compounds-(A) and (B). (Schema 2)

In 1936, Haworth reported that the product formed in this reaction is (B)[2] but later (1938) corrected this statement asserting that only (A) had been obtained[1]. Russell and others also surmized theoretically that the reaction of styrene oxide and malonic ester would form γ-phenylbutyrolactone corresponding to (A) thus proving this substance experimentally[3]. These facts indicate that it would be possible to obtain the objective substance by such a synthetic procedure, and the reactions were effected in the following manner with reference to procedure (1). (Schema 3)

Epoxide (II) of good purity was obtained in a fair yield by the use of perbenzoic acid and its boiling point agreed with that of (I). The condensate (III), formed by the reaction of epoxide (II) and ethyl sodiomalonate, was easily soluble in ethanol so that it was directly treated without isolation and, (IV) was obtained in a yield about three times as that obtained by Haworth. Demethylation was effected by the use of hydrogen bromide in acetic acid.

As the compound (V) thereby obtained was only sparingly soluble in carbon tetrachloride

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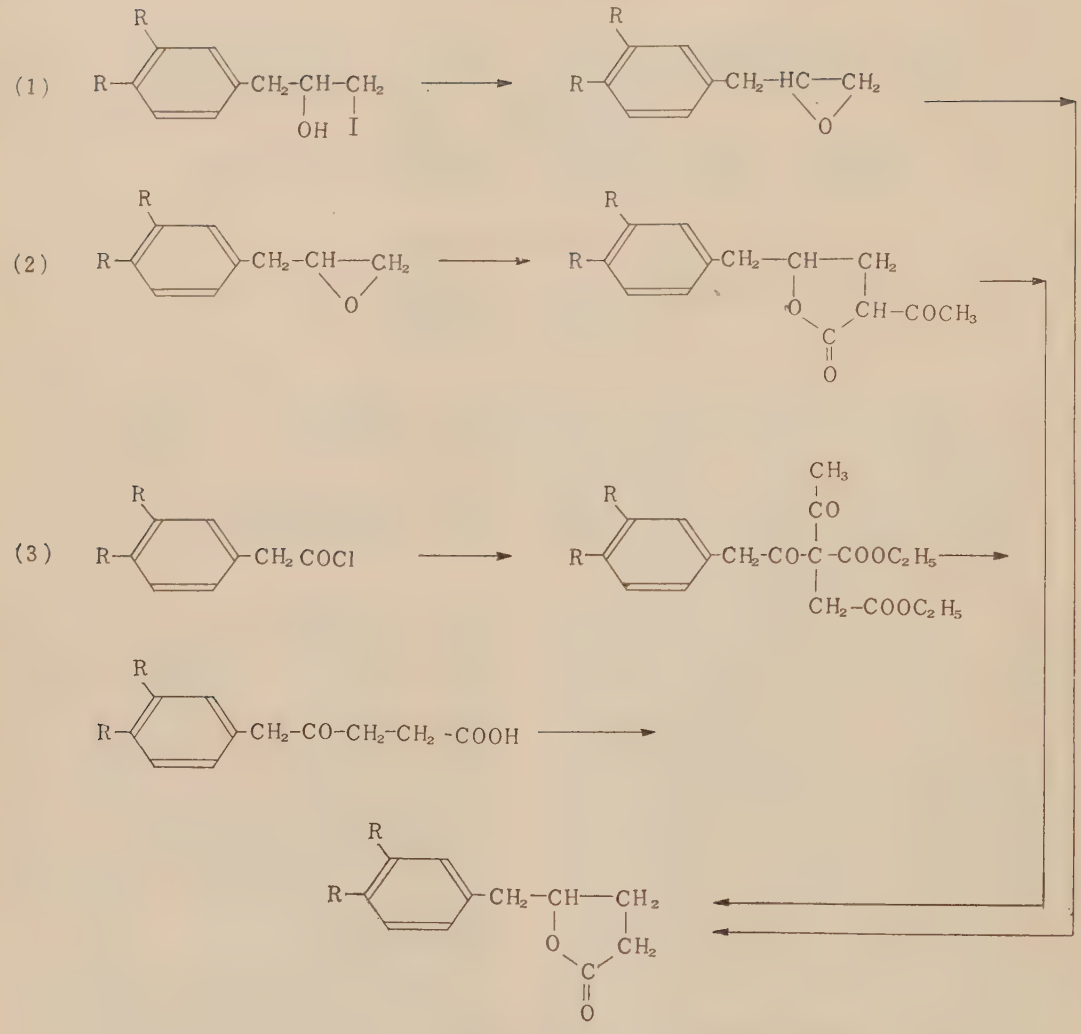
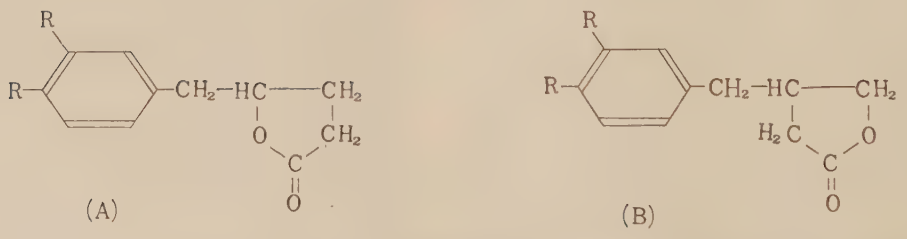
1) R.D. Haworth and J. Atkinson, *J. Chem. Soc.*, **1938**, 797

2) R.D. Haworth and T. Richardson, *J. Chem. Soc.*, **1936**, 348.

3) R.R. Russel and C.A. Vander, *J. Am. Chem. Soc.*, **69**, 11 (1947).



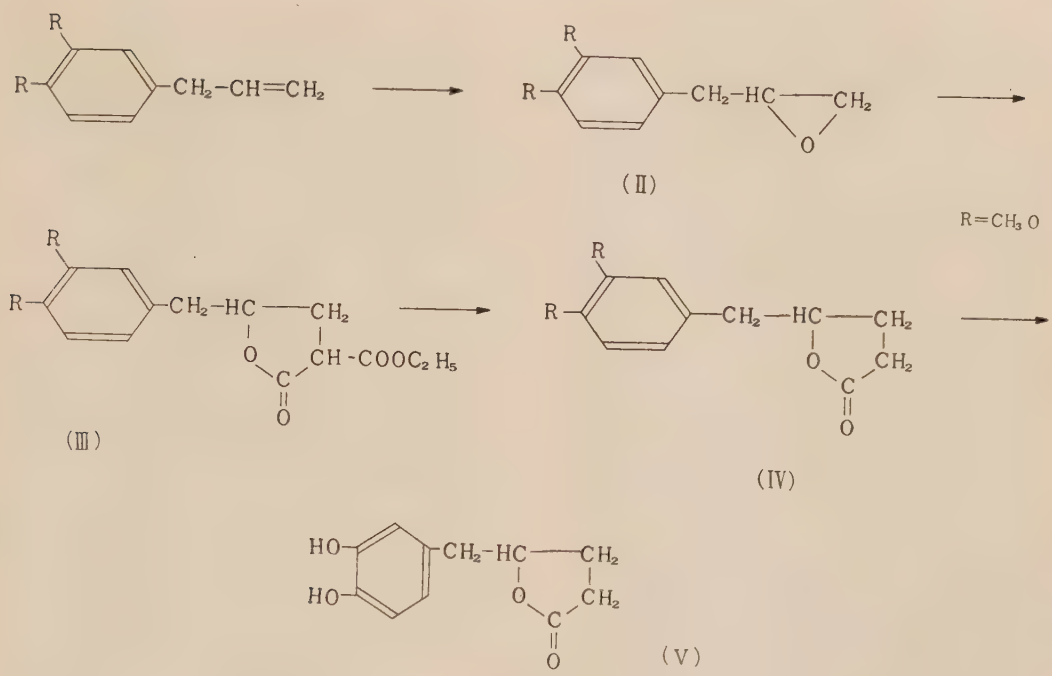
Schema 1. R = CH_3O



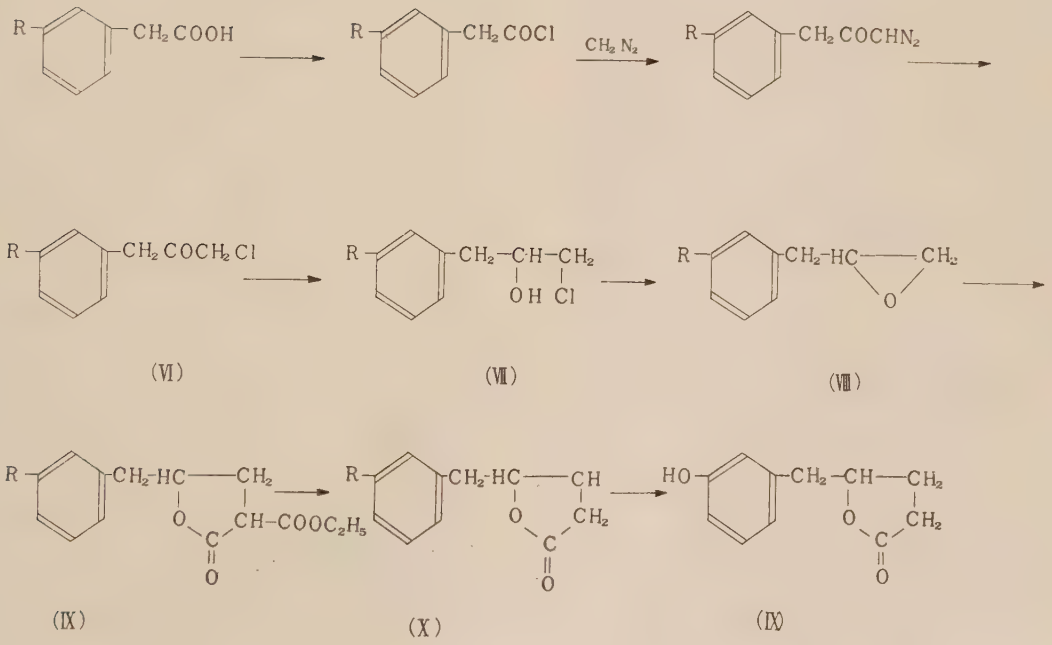
Schema 2.

it was led to the methyl derivative (IV) whose infrared absorption spectrum in carbon tetrachloride solution showed complete agreement with the dimethyl derivative of H, establishing the identity of these structures.

The syntheses of *dl*-δ-(3-methoxyphenyl)-γ-



Schema 3.



Schema 4.

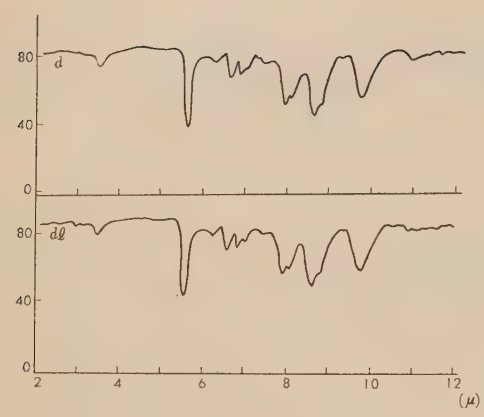


FIG. 1. Infrared Absorption Spectra of *d*- and *dl*-*δ*-(3,4-Dimethoxyphenyl)-*γ*-valerolactone (in CCl_4, NaCl prism)

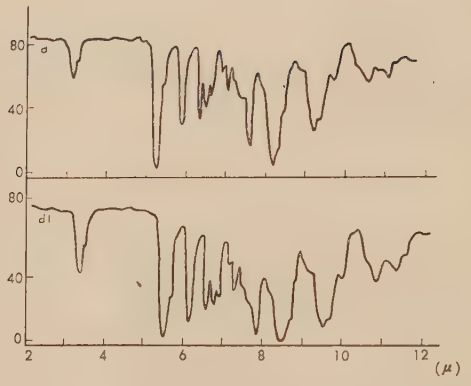


FIG. 2. Infrared Absorption Spectra of *d*- and *dl*-*δ*-(3-Methoxyphenyl)-*γ*-valerolactone (in CCl_4, NaCl prism)

valerolactone and its demethylated compound started with 3-methoxyphenylacetic acid since there was no substance corresponding to eugenol and followed the sequence shown in Schema 4.

3-Methoxybenzyl chloromethyl ketone (VI) was obtained from diazo ketone, formed from acid chloride and diazomethane in the usual manner, by the introduction of dry hydrogen chloride gas without isolation. Reduction of the carbonyl in (VI) to alcohol was effected with sodium borohydride without liberation of the chlorine atom and the epoxide (VIII) was prepared in the usual manner. The addition reaction of the epoxide and ethyl malonate followed the procedures adopted in the previous case but the sodium salt of (IX), insoluble in ethanol, was collected by filtration for further treatment. Demethylation in this case was solely effected by hydrogen bromide.

The boiling point of the compound (X) thereby obtained agreed with that of the methyl compound of G and its analytical values were also in good accord with the theoretical. Infrared spectra of both in carbon tetrachloride showed no difference in the absorption, thereby establishing the identity of these structures. The demethylated compound derived from (X) melted at 105-106° in both racemic and dextrorotatory compounds.

Consequently, the structure of G and H, the intermediate metabolites of catechin, were determined as *d*-*δ*-(3-hydroxyphenyl)-and *d*-*δ*-(3,4-dihydroxyphenyl)-*γ*-valerolactone.

EXPERIMENTAL

1, 2-Epoxy-3-(3, 4-dimethoxyphenyl)-propan (II). Fifty grams of O-methyl eugenol was added to a solution of 36.5 g of perbenzoic acid in 370 ml of chloroform at 0°. The solution was maintained at this temperature for fourty-eight hours, and benzoic acid was removed from the chloroform solution by shaking with several portions of ten per cent ammonium hydroxide solution. The chloroform solution was washed with water to remove ammonia, dried with anhydrous sodium sulfate and evaporated. By fractional distillation, it yielded 26.4 g of (II); b.p. 138–143°.

***δ*-(3, 4-Dimethoxyphenyl)-*γ*-valerolactone (IV).** To a solution of ethyl sodiomalonate prepared from 3.1 g of sodium and 21.8 g of ethyl malonate in 100 ml of ethanol, 26.4 g of (II) was added. Three days later, 100 ml of 10 per cent sodium hydroxide solution was added and the solution was refluxed for thirty minutes, and then ethanol was removed under reduced pressure. The reaction mixture was extracted with ether to remove the neutral substances, acidified with sulfuric acid and extracted with chloroform. The chloroform layer was dried with anhydrous sodium sulfate. After evaporating chloroform the residue was maintained at 130–140° until evolution of carbon dioxide ceased (in about one hour). Distillation gave 16.2 g of yellow oil, which slowly solidified. Recrystallization from etanher-methol

gave colorless needles; m.p. 83.5°–84.5°. *Anal.* Found: C, 66.44; H, 6.93. Calcd. for $C_{13}H_{16}O_4$: C, 66.08; H, 6.83.

δ-(3, 4-Dihydroxyphenyl)-γ-valerolactone (V). A mixture of 10 g of (IV), 20 ml of acetic acid and 80 ml of 47 per cent hydrobromic acid was refluxed gently for one hour and then acetic acid and hydrobromic acid were removed under reduced pressure. This oily residue was dissolved in 100 ml of water, neutrallized with sodium carbonate, extracted with ethyl acetate, dried with anhydrous sodium sulfate and the solvent was removed. Recrystallization was repeated from water and 2.1 g of colorless crystals was obtained, prisms; m.p. 132–133°. *Anal.* Found: C, 63.39; H, 6.04. Calcd. for $C_{11}H_{12}O_4$: C, 63.45; H, 5.81.

3-Methoxybenzylchloromethyl ketone (VI). To the 250 ml of ether solution of diazomethane (prepared from 20 ml of nitroso N-methyl urethane) 9.1 g of 3-methoxyphenyl acetylchloride was added dropwise and it was allowed to stand at room temperature. After standing for two hours the solution of diazoketone was cooled in ice and treated with dry gaseous hydrogen chloride until the passage of gas no longer caused evolution of nitrogen. The ether solution was washed with water and 5 per cent sodium carbonate solution, dried with calcium chloride, evaporated and the products were distilled as yellow liquid. The yield of Ketone (VI) was 8.4 g; b.p. 1, 108–110°. *Anal.* Found: C, 60.48; H, 5.54. Cacd. for $C_{10}H_{11}O_2Cl$: C, 60.40; H, 5.58.

1-Chloro-3-(3-methoxyphenyl)-2-propanol (VII). Seven grams of chloromethyl ketone (VI) in 20 ml of methanol was added to a solution of 1 g of sodium borohydride in 20 ml methanol at 0° with stirring, and maintained at this temperature for one hour and subsequently at room temperature for three hours. By the addition of 3 ml of acetic acid, the remained sodium borohydride was decomposed and the solvent was evaporated. The residue was dissolved by the addition of 50 ml of water, extracted with ether and the ether layer was dried, removed and distilled under reduced pressure. Yield 5.6 g of colorless liquid; b.p. 0.9, 108–110°. *Anal.* Found; C, 59.40; H, 6.21. Calcd. for $C_{10}H_{13}O_2Cl$: C, 59.86; H, 6.53.

1, 2-Epoxy-3-(3-methoxyphenyl)-propan (VIII). To a solution of 1.15 g of sodium hydroxide in 10 ml of water, 5.5 g of chloride (VII) was added and stirred vigorously for one hour at room temperature. Stirring was then stopped and the upper layer extracted with ether, the ether solution dried, evaporated and fractionated. Colorless liquid 4.4 g; b.p. 0.9, 80–84°. *Anal.* Found; C, 73.24; H, 7.39. Calcd. for $C_{10}H_{12}O_2$: C, 73.15; H. 7.31.

Ethyl-δ-(3-methoxyphenyl)-γ-valerolactone-α-carboxylate (IX). To a solution of ethyl sodiomalonate prepared from 0.6 g of sodium and 4.3 g of ethyl malonate in 20 ml of absolute ethanol, 4.3 g of oxide (VIII) was added and this was allowed to stand for twenty-four hours at room temperature. Crystals were filtered by suction, washed with ethanol, dissolved in water, acidified with hydrochloric acid and extracted with ether. The ether layer was dried with anhydrous sodium sulfate, evaporated and fractionated under reduced pressure. Yellow oil, 3.4 g; b.p. 0.8, 170–175°. *Anal.* Found: C, 64.27; H, 6.68. Calcd. for $C_{15}H_{18}O_5$: C, 64.74; H, 6.52.

δ-(3-Methoxyphenyl)-γ-valerolactone (X). A mixture of 15 ml of 10 per cent sodium hydroxide solution and 3 g of ester (IX) was heated at 80–90° for thirty minutes with stirring. This reaction mixture was acidified with hydrochloric acid and extracted with chloroform. The chloroform layer was dried with anhydrous sodium sulfate and evaporated. The residue was maintained at 130–140° until the evolution of carbon dioxide completed (in about one hour). By fractional distillation 2.1 g of colorless oil was obtained; b.p. 0.8 146–148°. *Anal.* Found: C, 69.60; H, 6.68. Calcd. for $C_{12}H_{14}O_3$: C, 69.88; H, 6.84.

δ-(3-Hydroxyphenyl)-γ-valerolactone (XI). Two grams of lactone (X) was dissolved in 10 ml of 47 per cent hydrobromic acid and refluxed for thirty minutes gently. The same procedure as (V) was carried out in this experiment with recrystallization from benzol. Yield 0.7 g of prisms; m.p. 105–106°. *Anal.* Found: C, 68.40; H. 6.12. Calcd. for $C_{11}H_{12}O_3$: C, 68.73; H, 6.29.

The author expresses his deep gratitude to Prof. Y. Oshima for his helpful guidance throughout this work, and to Dr. M. Fujisawa and Dr. N. Sugimoto of the Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd., for their kind encouragement. He is also indebted to Mr. M. Shido of the elemental analysis service center of Kyushu University for microanalyses and to Mr. K. Kodera of the Osaka Research Laboratory for the infrared analyses.

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The Chemical Structure of the Intermediate Metabolites of Catechin. IV

Structure of the Intermediate Metabolite (F)

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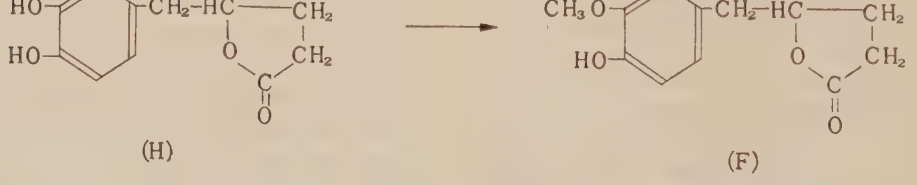
It has been reported that three neutral substances (F, G and H) are excreted into the urine after the oral administration of (+)-catechin to the rabbit, and also that substances G and H are respectively determined as δ-(3-hydroxyphenyl) and δ-(3, 4-dihydroxyphenyl)-γ-valerolactones. Considering the structures of G and H and the metabolic mechanism of phenols, the structure of F was assumed to be δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone and this compound was synthesized. Comparison of the natural and synthesized substances by paper partition chromatography established their identity and the structure of F was thereby confirmed.

It has already been shown[1] that three kinds of neutral substances (F, G and H) are excreted into the urine after oral administration of (+)-catechin to the rabbit. Substances G and H were obtained in a comparatively large amount and isolated in a crystalline state. Detailed studies on these substances established their chemical structure as δ-(3-hydroxyphenyl)- and δ-(3,4-dihydroxyphenyl)-γ-valerolactones. Substance F was obtained in a small amount and was not isolated in the crystalline state.

But from the findings 1) substance F showed a characteristic pink color reaction to diazotized benzene sufanilic acid and sodium carbonate, a color reaction similar to that of eugenol and substances of 3-methoxy-4-hydroxyphenyl acetic acid series, 2) and from their R_F values in paper chromatography with various solvents, 3) protocatechuic, vanillic and 3-hydroxybenzoic acids were excreted in the urine of rabbits after administration of catechin[2] and the neutral substances, G and H, were considered to be the precursors of hydroxybenzoic acid and protocatechuic acid, and it was consequently considered that F might be a precursor of vanillic acid, and 4) compounds possessing hydroxyls in 3- and 4-positions in the benzene rings like H are methylated at 3-position[3], the structure of F was therefore assumed to be δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone.



(H) (F)

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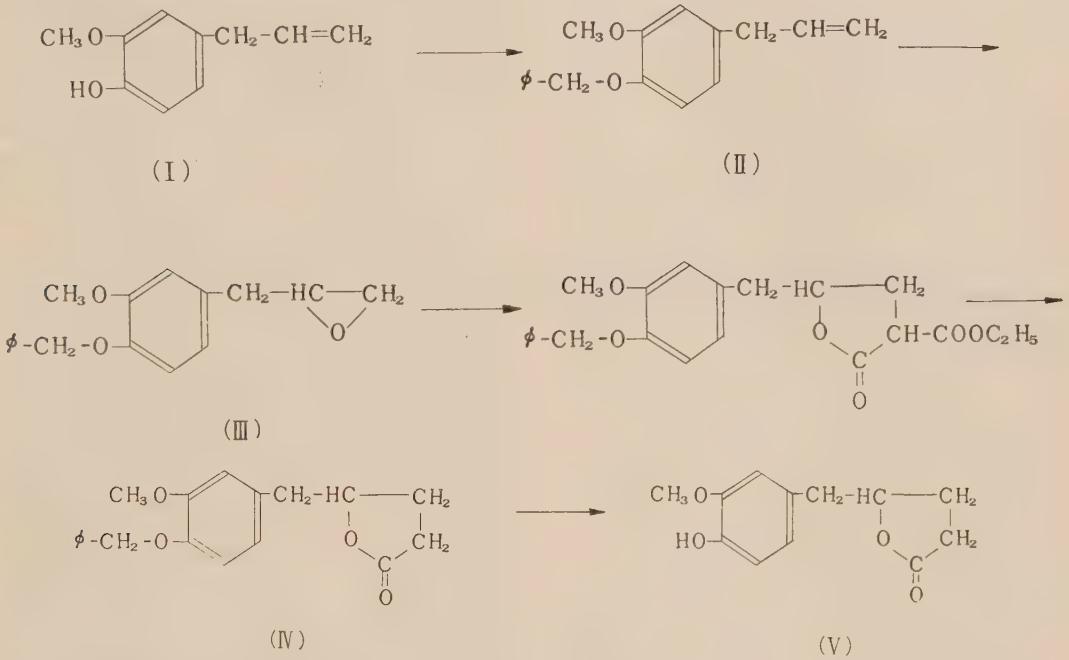
1) Y. Oshima and H. Watanabe, *J. Biochem.* (*Japan*), **45**, 973 (1958).

2) Y. Oshima and H. Watanabe, *J. Biochem.* (*Japan*), **45**, 861 (1958).

3) F. DeEds, A.N. Booth, and F.T. Jones, *J. Biol. Chem.*, **225**, 615 (1957).

Hence this compound was synthesized by the route shown below:



The procedure of this synthesis is similar to that for the preparation of H, described in Part III of this series but the condensation of ethyl malonate and the epoxide (III) was considered to be difficult if a hydroxyl is present and the latter is led to a benzyl derivative. Similar to the case of H, the reaction proceeded smoothly and liberation of the benzyl group was effected by catalytic reduction under usual conditions.

Paper partition chromatography was carried out on this δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone thus obtained and on F, purified by column chromatography over powdered filter paper, using solvent systems of benzene-acetic acid-water (water-saturated benzene+10 per cent acetic acid), butanol-water (water-saturated butanol), and 10 per cent acetic acid. R_F values of the chromatogram are indicated in Table I.

As it was made clear from Table I, F and F′ were considered to be the same substance. Optical rotation of this substance was assumed to be dextrorotatory, as in G and H, and the chemical structure of F was considered to be *d*-δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone.

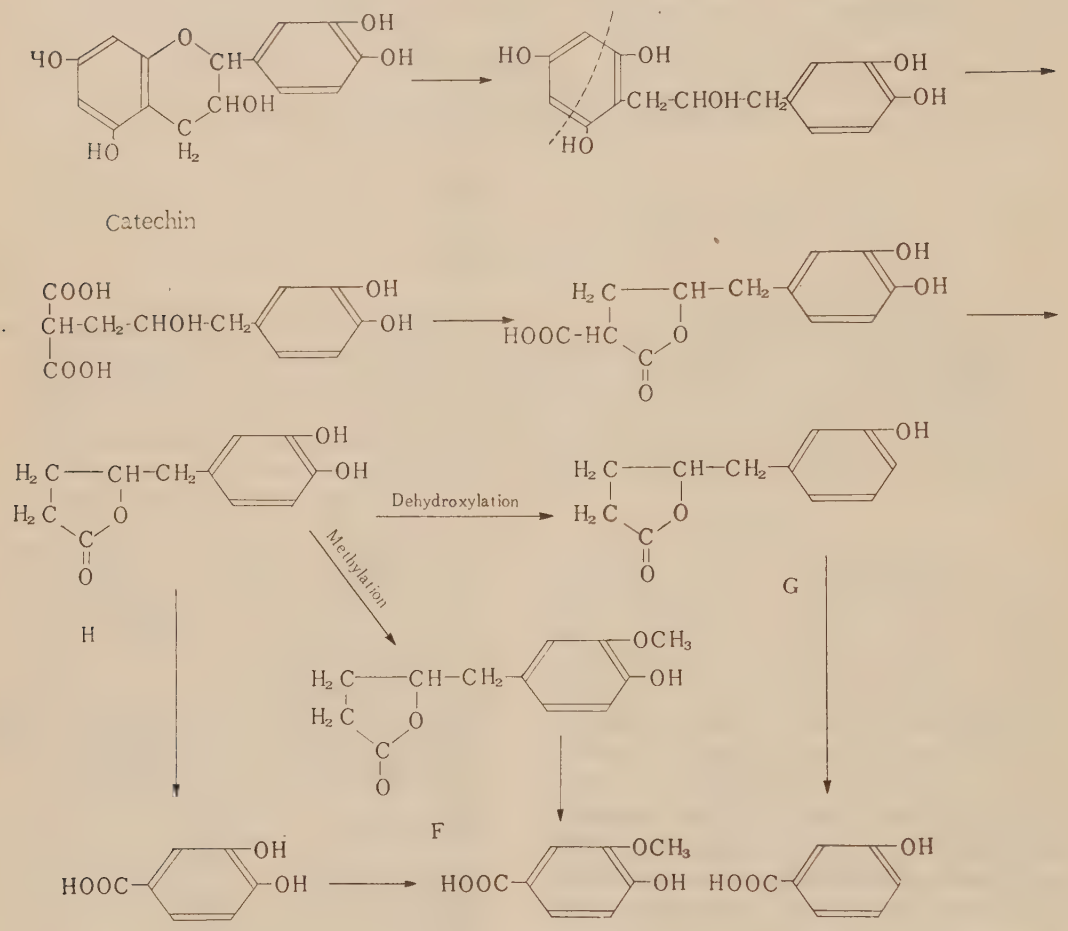
Table I. R_F Values of F and δ-(3-Methoxy-4-Hydroxyphenyl)-γ-Valerolactone by Paper Partition Chromatography

Solvent Systems	R_F Values	
	F′ (synth.)	F (natural)
Benzene-AcOH-H_2O	0.94	0.95
BuOH-H_2O	0.89	0.89
10% AcOH	0.79	0.77

Thus, the chemical structures of the neutral intermediate metabolitis (F, and H) of (+)-catechin were respectively determined, and the acidic intermediate metabolites were reported to be protocatechuic, vanillic and 3-hydroxy benzoic acids in another report[2].

Therefore, judging from these intermediates the chemical pathway of (+)-catechin were assumed to be the same as those in Table II.

TABLE II. METABOLIC PATHWAYS OF (+)-CATECHIN IN RABBITS



The biochemical mechanisms are under study in our laboratory in detail.

EXPERIMENTAL

O-Benzyl eugenol (II). Sodium eugenolate perpared from 4.6 g of sodium and 32.8 g of eugenol (I) in 100 ml of absolute ethanol was mixed with 32.3 g of benzyl chloride and refluxed for five hours gently. After cooling, sodium chloride was filtered by suction and ethanol was evaporated. To this residue 50 ml of water was added and the separated oily substances were extracted with ether. The ether layer was shaken with 10 per cent sodium hydroxide solution and washed with water and then dried with sodium sulfate.

After removing ether, fractional distillation yielded 30.1 g of yellow liquid; b.p. 0.8 143–146°. *Anal.* Found: C, 80.34; H, 7.39. Calcd. for $C_{17}H_{18}O_2$: C, 80.28; H, 7.13.

1, 2-Epoxy-3-(3-methoxy-4-benzoxyphenyl)-propan (III). To a solution of this substance 11.0 g of (II) was added at 0°. The solution was maintained at this temperature for fourty-eight hours and then benzoic acid was removed from the chloroform solution by shaking with several portions of 10 per cent sodium hydroxide solution. The chloroform solution was washed with water to removed the remaining alkali and dried with anhydrous sodium sulfate. Fractional distillation yielded 9.1 g of yellow liquid; b.p. 0.8 168–172°. *Anal.* Found: C, 75.25; H, 6.72. Calcd. for $C_{17}H_{18}O_3$: C, 75.52; H, 6.71.

δ-(3-Methoxy-4-benzoxyphenyl)-γ-valerolactone (IV). To a solution of ethyl sodiomalonate prepared from 0.75 g of sodium and 5.2 g of ethyl malonate in 20 ml of absolute ethnol, 8.8 g of epoxide (III) was added and it was allowed to stand at room temperature. After standing for twenty-four hours, 30 ml of 10 per cent sodium hydroxide solution was added and after being refluxed for thirty minutes gently, ethanol was removed under reduced pressure. The reaction mixture was extracted with ether in order to remove the neutral substances. The alkaline layer was acidified with sulfuric acid and extracted with ether. The ether layer was washed with water, dried, evaporated and fractionated under reduced pressure; 4.4 g of yellow oil was obtained. b.p. 0.8 218–222°. *Anal.* Found: C, 73.01; H, 6.46. Calcd. for $C_{19}H_{20}O_4$: C, 73.05; H, 6.45.

δ-(3-Methoxy-4-hydroxyphenyl)-γ-valerolactone (V). In 20 ml of absolute ethanol 3.7 g of (IV) was dissolved and 0.2 g of Adams' platinum oxide was added. This mixture was catalytically reduced at room temperature (30°). After four hours 340 ml of hydrogen was absorbed and platinium black was filtered. The filtrate was concentrated and the residue was fractionated under reduced pressure, and 1.8 g of a semi-solid substance which soon solidified was obtained; b.p. 0.8, 191–196°. After recrystallization from benzol this substance melted at 108–109°. Colorless pillars. *Anal.* Found: C, 64.88; H, 6.65. Calcd. for $C_{12}H_{14}O_4$: C, 64.84; H, 6.49.

Extraction and purification of F. As described in the previous paper 32.4 l of urine of rabbits administered 200 g of (+)-catechin was collected and H and G were isolated in crystalline states. The filtrate from which G was removed was alkalized with sodium hydroxide solution and neutral substances were extracted with ether. The alkaline layer was acidified with hydrochloric acid solution and the phenolic substances separated were extracted with ethyl acetate. The ethyl acetate layer was dried with anhydrous sodium sulfate and evaporated an oily residue remaining. Purification of F was performed, using a column in which cellulose powder was uniformly packed in a glass tube of 2.2× 60 cm. The residue was placed on the top of the column and eluted with a mixture of water saturated benzene and acetic acid (9 : 1 by volume) and fractionated. The fractions which showed a pink-red color reaction with diazotized benzene sulfanilic acid and 20 per cent sodium carbonate solution were combined and the solvent was evaporated. Small amounts of semisolid substances were obtained.

Paper partition chromatography of δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone (F′) and F. Paper partition chromatography of δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone (F′) and F was performed using a filter paper of Toyo Roshi No. 50 and the following three solvents.

(a) Water saturated benzene and acetic acid (9 : 1 by volume).
(b) Water saturated *n*-butanol.
(c) Ten per cent acetic acid solution.

After the development of 28–32 cm in length the paper was dried and the colour was developed by spraying freshly prepared diazotized sulfanilic acid followed by 20 per cent sodium carbonate solution. The R_F values were compared and the results are shown in Table 1.

The author expresses his deep gratitude to Prof. Y. Oshima for his helpful guidance throughout this work, and to Dr. M. Fujisawa and Dr. N. Sugimoto of the Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd. for their kind encouragement. He is also indebted to Mr. M. Shido of the elemental analysis service center of Kyushu University for the microanalysis.

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Notes on the Colorimetric Determination of Inorganic Orthophosphate

Part I. Determination of Inorganic Orthophosphate in the Presence of Some Acid-labile Phosphate Compounds of Biochemical Significance*

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The effect of the amount of the amidol reagent on the rates of hydrolysis of some acid-labile phosphate compounds in the determination of inorganic orthophosphate according to the method of Allen[2] and its modifications by Whelan and Bailey[9] and by Nakamura[12] is reported. It is shown in this paper that the most important factor in these methods is the temperature at which molybdenum blue color is developed. The rate constants of acid hydrolysis of adenosine triphosphate, glucose-1-phosphate, fructose-1,6-diphosphate, phosphoryl (enol) pyruvate and inorganic pyrophosphate at various temperatures under the experimental conditions of Nakamura's method in the presence and absence of molybdate are also presented.

Many analytical methods have been reported for the determination of P_i**. Among these, the colorimetric methods of Fiske and Subbarow[1] and Allen[2] have most frequently been employed for biological materials. In these methods, P_i is converted to phosphomolybdic acid and then reduced to molybdenum blue in acid media, and the color intensity is measured in a colorimeter. Owing to high acidities of the colored solutions (approximately 0.5 N sulfuric acid or perchloric acid), acid-labile phosphate compounds are more or less easily hydrolyzed to form P_i which interferes with the determination[3,4]. Thus, these methods are inapplicable for the determination of P_i in the presence of, for example, creatine phosphate or deoxyribose-1-phosphate, which are completely hydrolyzed to P_i under the conditions of these methods. Other acid-labile phosphate compounds interfere more or less seriously according to their labilities in the acid media[4].

The true values of P_i in the presence of such acid-labile phosphate compounds can be determined by several procedures. To minimize hydrolysis of acid-labile phosphate compounds, Lowrey and López[5] reduced phosphomolybdic acid in an acetate buffer of pH 4.0, using ascorbic acid as a reducing agent. This method has a disadvantage because the color is unstable and susceptible to many interfering substances. Berenblum and Chain[6] extracted phosphomolybdic acid formed in acid media with isobutanol and molybdenum blue color was developed in the isobutanol layer. Several modifications of this method have been proposed[7,8]. Although these methods give more accurate values of P_i than those using acid

* A preliminary report of a part of this paper appeared elsewhere [M. Nakamura and K. Mori, *Nature*, **182**, 1441 (1958)].

** The following abbreviations are used throughout this paper: Pi, inorganic orthophosphate; G-1-P, α-D-glucose-1-phosphate; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphate, respectively; FDP. fructose-1,6-diphosphate; PEP. phosphoryl (enol)-pyruvate; A_7P, phosphate liberated as Pi by heating in 1N sulfuric acid for 7 minutes in a vigorously boiling water bath.

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3) O. Lindberg and L. Ernster, in D. Glick (ed.), Methods of Biochemical Analysis, Vol. III, p. 1. Interscience Publishers (New York, 1956).
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5) O. H. Lowrey and J.A. López, *J. Biol. Chem.*, **162**, 421 (1946).
6) I. Berenblum and E. Chain, *Biochem. J.*, **32**, 295 (1938).
7) J. B. Martin and D. M. Doty, *Anal. Chem.* **21**, 965 (1949).

media for color development, they are more complicated and time-consuming. Accordingly, it is not practical to employ these methods as a routine procedure.

During their studies on the action pattern of amylophosphorylase, Whelan and Bailey[9] found that in the determination of P_i according to the method of Allen in the presence of a large amount of G-1-P, the rate of hydrolysis of G-1-P could be reduced to approximately one-seventh of that in the original method by halving the specified amount of the amidol reagent. Some reports[10,11] have been published on the catalytic effect of molybdate upon the rate of hydrolysis of some phosphate compounds by acid, but the catalytic effect of amidol has so far not been reported. Moreover, one of us[12] has, several years ago, developed a modified procedure of Allen, in which sulfuric acid was used in place of perchloric acid and the amount of the amidol reagent was halved. At that time, the amount of the amidol reagent was halved simply because half of the original quantity was found sufficient for most determinations and thus excess of the reagent might better be avoided. A possible effect of amidol on the rate of hydrolysis of acid-labile phosphate compounds was not examined.

It may therefore be worthwhile to reexamine the catalytic effect of the amidol reagent on the rate of acid hydrolysis of some phosphate compounds by the original method of Allen and by the modified procedures of Whelan and Bailey and of Nakamura.

EXPERIMENTAL

Materials

G-1-P: Crystalline potassium salt of G-1-P was prepared essentially by the method of Hassid and McCready[13]. Amberlite IR-112 (H form) and Amberlite IR-4B (acetate form[14]) resins were used as cation and anion exchangers, respectively. Crude crystals were dissolved in a small volume of water and the solution was treated with activated charcoal. An equal volume of 95% ethanol was added to the filtered solution, and the mixture was allowed to stand overnight in a refrigerator to complete crystallization. Purity, determined by the amount of Δ_7-P, was 97.1% of the theoretical value calculated as $C_6H_{11}O_9PK_2 \cdot 2H_2O$.

ATP: Barium salt of ATP was prepared from rabbit muscle by the method of Dounce et al.[15]. It was further purified by ion-exchange chromatography according to the procedure of Cohn and Carter[16]. The purified product was stored as the dried barium salt. Purity, determined by the values of Δ_7-P and ultraviolet absorption at 259 mμ[17], was 90.1% of the theoretical value calculated as $C_{10}H_{12}O_{13}N_5P_3Ba_2 \cdot 4H_2O$.

Sodium pyrophosphate: Commercial sodium pyrophosphate (C.P. grade) was used without further purification. Purity, determined by the amount of Δ_7-P, was 99.0% of the theoretical value calculated as $Na_4P_2O_7 \cdot 10H_2O$.

FDP: Preparation and purification of the acid barium salt of FDP will be described elsewhere*. Purity, determined by the amount of total phosphorus and fructose[18], was 91.6% of the theoretical value calculated as $C_6H_{12}O_{12}P_2Ba \cdot H_2O$.

PEP: Silver barium salt of PEP was synthesized by the method of Baer[19]. Purity, determined by the amount of hypoiodite-labile phosphorus, was 98.0% of the theoretical value calculated as $C_3H_2O_6PAgBa \cdot 2H_2O$.

ATP, FDP, and PEP were used in the form of sodium salts.

Solutions

Perchloric acid: 60% (w/v), extra pure grade.

Sulfuric acid: 15% (v/v), C.P. grade.

Ammonium molybdate reagent: 3.3% in water.

Amidol reagent: Four hundred mg of amidol (2,4-

8) H. Takahashi, *Seikagaku* (J. Japan. Biochem. Soc.), **26**, 690 (1955).
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13) W. Z. Hassid and R. M. McCready, in Biochemical Preparations, Vol. IV, p. 63. John Wiley & Sons (New York, 1955).
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19) E. Baer, in Biochemical Preparations, Vol. II, p. 25. John Wiley & Sons (New York, 1952).

diaminophenol hydrochloride) and 8 g of sodium bisulfite were dissolved in 100 ml of water. In case the solution was colored, it was decolorized with a small amount of activated charcoal. This reagent may be kept for several weeks in a well-stoppered dark bottle in a refrigerator.

Standard orthophosphate solution: About 43 mg of accurately weighed pure potassium dihydrogen phosphate was dissolved in 100 ml of water.

Procedures

For experimental convenience, the original procedure of Allen was somewhat modified, although agreeing exactly with the original method in the final concentrations of the reagents. One hundred ml of the reaction mixture contained 8 ml of 60% perchloric acid, 20 ml of the amidol reagent, 10 ml of 3.3% molybdate reagent, 0.5 or 2.0 ml of the standard phosphate solution, and a specified amount of a phosphate compound to be tested. In the modification made by Whelan and Bailey, the amount of the amidol reagent was halved, i.e., 10ml. In Nakamura's modification, 10 ml of 15% sulfuric acid was substituted for perchloric acid and the quantity of the amidol reagent was 10 ml. A series of experiments with 20 ml of the amidol reagent was also carried out for the sake of comparison.

The reaction mixtures, contained in 200-ml Erlenmeyer flasks, were kept in a well-regulated water bath at 18° or 33°. At 5 or 10 min. intervals, aliquots were withdrawn and color intensities were measured in a 1-cm cuvette using a Hitachi model EPO-A photoelectric colorimeter fitted with filter R (maximum absorption at 660 mμ).

Rates of hydrolysis of some acid-labile phosphate compounds.

Reaction mixture: Ten ml of the reaction mixture contained 1 ml of 15% sulfuric acid and 8~16 μ moles of the phosphate compound subjected to examination. In another series of experiments, which was intended to examine the effect of molybdate, 1 ml of 3.3% molybdate reagent was also included.

Measurement of the rate of hydrolysis: The reaction mixtures prepared as described above were kept at a constant temperature (27°, 37°, 47°, or 57°C) in a well-regulated water bath. At intervals, 1-ml aliquots were withdrawn for the determination of P_i.

Determination of inorganic orthophosphate: To give the true values of P_i, Takahashi's modification[8] of Berenblum and Chain's method was employed. In this method, at the stage of extraction of phosphomolybdic acid with isobutanol, small particles of water that remained in the isobutanol layer often caused significant errors. Therefore, to overcome this difficulty, the isobutanol layer was centrifuged to remove the water particles, and an aliquot of the upper isobutanol layer was withdrawn to develop color with ascorbic acid.

RESULTS

Effect of hydrolysis of phosphate compounds on the determination of P_i.

The increase in color intensity during the course of 100 min. at 33° and 18° in the presence of a small amount of P_i (0.31μ mole P/ml) and one of the phosphate compounds to be tested is shown in Fig. 1-I and -II, respectively. Results obtained from a larger amount (20 ml) of the amidol reagent in combination with sulfuric acid were omitted. Further, results obtained from larger quantities of P_i (1.24μ moles P/ml) were also omitted, because essentially similar results as those given in Fig. 1 were obtained.

As can be seen in the figure, the amount of the amidol reagent had only a slight effect on the rate of acid hydrolysis of some acid-labile phosphate compounds excluding ATP. Thus, the finding of Whelan and Bailey that hydrolysis of G-1-P could be considerably reduced by reducing the amount of the amidol reagent to one-half of that used in Allen's method could not be confirmed.

From the figure, it is obvious that the most important factor involved is the temperature at which molybdenum blue color is developed. At 33°, there was a remarkable increase in color intensity, particularly in the presence of G-1-P and ATP, while at 18°, even G-1-P and ATP were not hydrolyzed to a significant degree. As both ATP and G-1-P are moderately labile and there are many occasions in biochemical works to determine P_i in the presence of these compounds, the increase in color intensity caused by the hydrolysis of either ATP or G-1-P under various conditions is given in Figs. 2 and 3, respectively. As can be seen in Fig. 2, more ATP was hydrolyzed at 33° in sulfuric acid medium when a larger

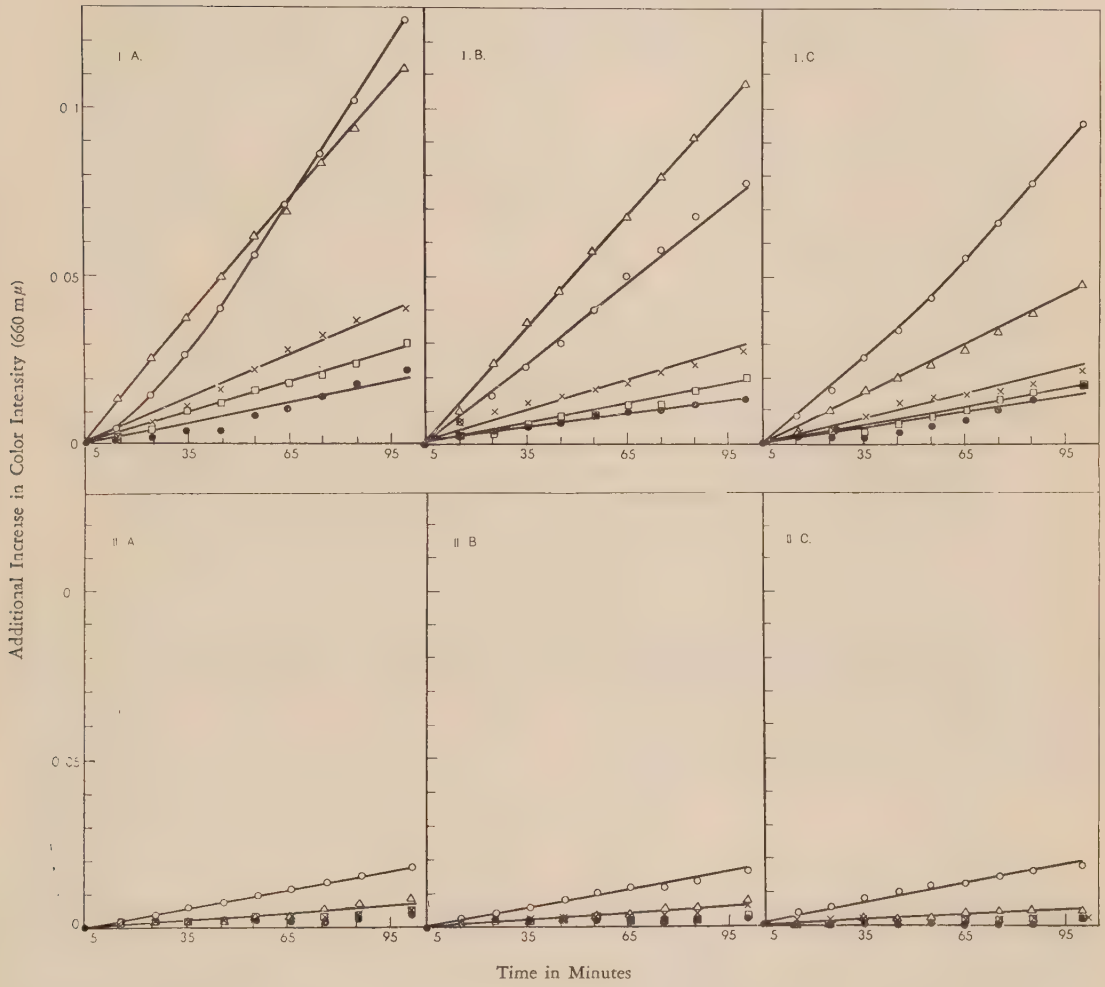

FIG. 1. Additional Increase in Color Intensity in the Determination of Inorganic Orthosphosphate (0.31 μ moles P/ml) Due to Hydrolysis of Coexistent Acid-labile Phosphate Compounds by Various Methods.

I: Molybdenum blue color was developed at 33±0.1°.

II: Molybdenum blue color was developed at 18±0.1°.

A. Allen's method.---Eight ml of 60% perchloric acid, 20 ml of the amidol reagent, and 10 ml of 3.3% molybdate reagent, in a total volume of 100 ml.

B. Whelan and Bailey's modification.---Eight ml of 60% perchloric acid, 10 ml of the amidol reagent, and 10 ml of 3.3% molybdate in a total volume of 100 ml.

C. Nakamura's modification.---Ten ml of 15% sulfuric acid, 10 ml of the amidol reagent, and 10 ml of 3.3% molybdate in a final volume of 100 ml.

The reaction mixtures contain, in addition: ○, 63 μmoles of ATP; △, 114 μmoles of G-1-P; ×, 73 μmoles of FDP; □, 115 μmoles of PEP; ●, no addition.

The color intensity at 5 min after color development was arbitrarily taken as zero.

amount of the amidol reagent was present in the reaction mixture. The amount of amidol had only a small effect in perchloric acid medium. Fig. 3 shows that more G-1-P was hydrolyzed at 33° in perchloric acid than in sulfuric acid and the amount of amidol had

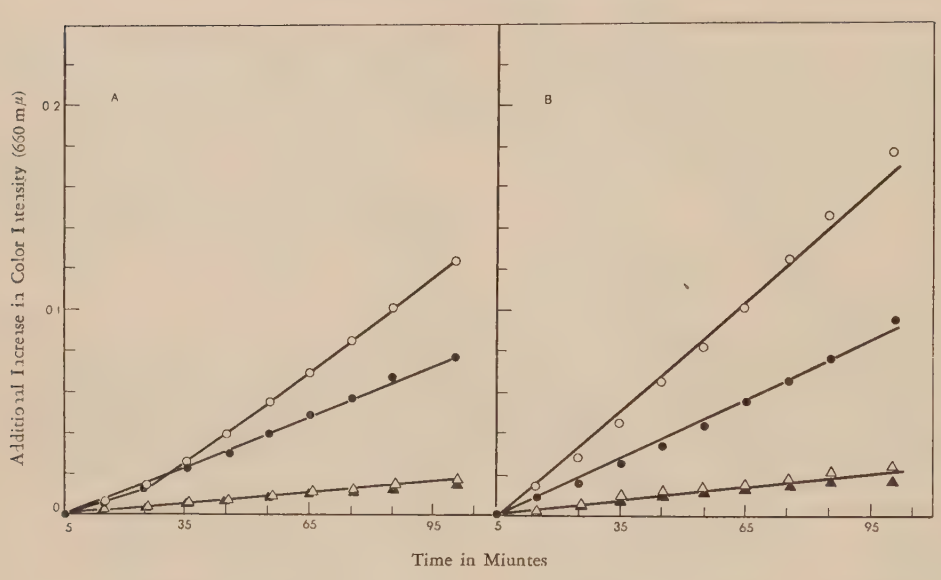

FIG. 2. Additional Increase in Color Intensity Due to Hydrolysis of ATP (63 μ moles/ml).

A: Eight ml of 60% perchloric acid and 10 ml of 3.3% ammonium molybdate in 100 ml.
B: Ten ml of 15% sulfuric acid and 10 ml of 3.3% molybdate in 100 ml.
Circles, color developed at 33°; triangles, at 18°. Open signs, with 20 ml of the amidol reagent; closed signs, with 10 ml of the amidol reagent.

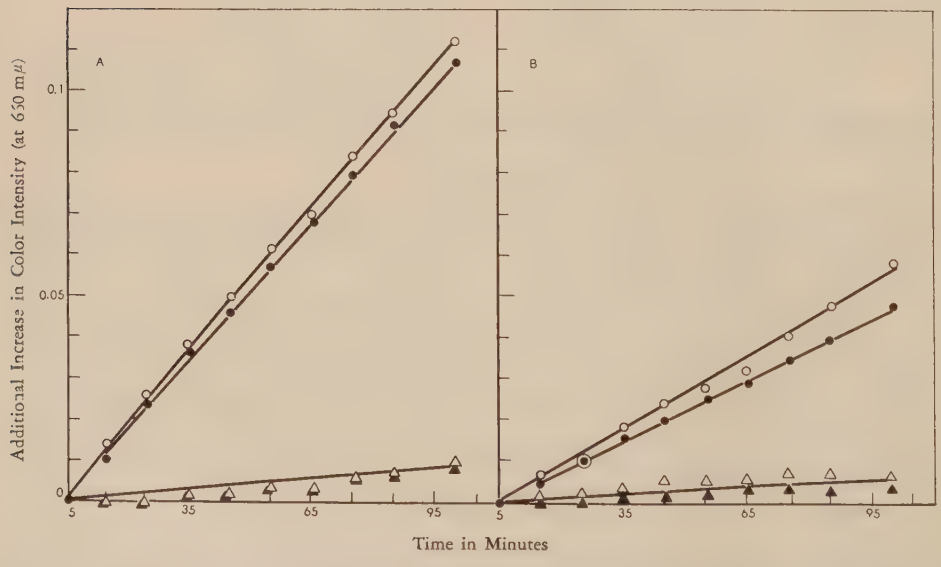

FIG. 3. Additional Increase in Color Intensity Due to Hydrolysis of G-1-P (114 μ moles/ml).

For experimental details, see the lengend of Fig. 2.

TABLE I. RATE OF HYDROLYSIS OF SOME ACID-LABILE PHOSPHATE COMPOUNDS

On assuming that acid hydrolysis of the following compounds given in the table follow the first-order reaction kinetics, first-order rate constants are given here. Figures in the table are expressed in units of 10^{-4} min^{-1}.

Temperature	27°		37°		47°		57°	
Molybdate*	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)
G-1-P	7.50	4.99	29.4	23.0	113	85.7		
ATP[1]	2.11	3.01	6.43	8.63	15.3	28.8		
$Na_4P_2O_7$	2.21	2.78	4.60	6.65	12.1	21.1		
FDP[2]					2.16	1.87	5.95	5.18
PEP					2.96	4.77	9.74	14.2

* (+), Presence of molybdate; (−), absence of molybdate.

1). The initial concentration of ATP was calculated from the sum of two acid-labile phosphorus atoms. The acid hydrolysis of ATP does not exactly fit the first-order kinetics, because hydrolysis of ATP is constituted from two successive reactions: (a) $ATP \xrightarrow{k_1} ADP + Pi$, and (b) $ADP \xrightarrow{k_2} AMP + Pi$. Although the values of k_1 and k_2 may be obtained by calculation (see K. Lohmann [*Biochem Z.*, **254**, 381 (1932)] and Weil-Malherbe and Green[10]), attempts to obtain these values were not made, since the determination of P*i* in these experiments are not completely accurate to serve for such a calculation.

2). One-half of the total phosphorus initially present in FDP was used in the calculation of the rate constant, because the phosphate group at carbon 6 of fructose is fairly resistant to acid.

only a slight effect. At 18°, hydrolysis of G-1-P and ATP was very slow irrespective of the nature of the acid and the amount of the amidol reagent.

Rate of hydrolysis of some acid-labile phosphate compounds.

It was shown in the preceding section that temperature is an important factor in the determination of P_i in the presence of acid-labile phosphate compounds. The results indicate, moreover, that acid labilities of these compounds differ very widely from one to another according to the nature of the substance. It was interesting, therefore, to determine the rate of hydrolysis of these compounds at various temperatures under conditions appropriate for the determination of P_i by Nakamura's procedure.

The results of such experiments are given in Table I. It can be seen that ATP, G-1-P, and pyrophosphate are far more labile than FDP and PEP. Molybdate showed an accelerating effect on the hydrolysis of ATP, pyrophosphate, and PEP, while it retarded the hydrolysis of G-1-P and FDP. The retarding effect of molybdate on the acid hydrolysis of G-1-P confirms the results of Weil-Malherbe and Green[10]. The presence of molybdate, however, had only a slight effect on the apparent activation-energy of the acid hydrolysis of these compounds.

DISCUSSION

The results obtained above show that at a higher temperature acid-labile phosphate compounds are more or less easily hydrolyzed and, as a consequence, may interfere with the determination of P_i by the method of Allen and the modifications of this method by Whelan and Bailey and by Nakamura. Such a difficulty can be overcome when determinations are carried out at a lower temperature. However, the rate of development of molybdenum blue color also slows down at a lower temperature. Keeping all these factors in mind, it is recommended that phosphorus determinations may be carried out at 18~20°. At this temperature, the rate of color development is rapid enough for practical use (5 to 10 min.) and the developed color is relatively stable for

a long period. At the same time, the interference by acid-labile phosphate compounds such as ATP and G-1-P is negligible in ordinary determinations.

The amount of the amidol reagent has some catalytic effect on the acid hydrolysis of ATP and G-1-P, although this is not so remarkable as reported by Whelan and Bailey. Therefore, the amount of amidol may better be reduced to one-half of the original method of Allen. Although the rate of hydrolysis of ATP was essentially the same either in sulfuric acid or in perchloric acid media, that of G-1-P was much faster in perchloric acid than in sulfuric acid. As the acidity and other conditions, except the nature of the acid used, were the same, the difference in the rate of hydrolysis G-1-P in both media is a problem to be elucidated in a future study.

Acknowledgment The authors wish to express their sincere thanks to Prof. S. Funahashi for his interest in this work and to Dr. T. Kôno for his valuable discussion.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 4, p. 278~280, 1959]

Notes on the Colorimetric Determination of Inorganic Orthophosphate

Part II. Determination of Total Phosphorus

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Received September 25, 1958

In the determination of total phosphorus, there was only a very slight, if any, condensation of orthophosphate to polyphosphates when the sample to be tested was incinerated in perchloric acid or in sulfuric acid. Both colorimetric method and radioactivity measurements were applied for the determination of orthophosphate remained as such in the digest. Also, the loss of phosphoric acid was not observed when care was taken so as not to allow the white vapor of the acid emerge from the digestion flask during incineration.

In the determination of the total phosphorus content of an organic substance, the sample must be first digested with perchloric acid or sulfuric acid with an oxidizing agent such as hydrogen peroxide or nitric acid, if necessary. After this wet incineration, inorganic orthophosphate (P_i) in the digest is determined by a proper method to give the total phosphorus content of the sample in question[1,2].

It is said that by this procedure a smaller value for total phosphorus may be obtained, because the sample is kept at a high temperature for a long time during the incineration, and as a consequence, P_i condenses to polyphosphates, which do not develop a molybdenum blue color in the usual method employed. Therefore, the determination of P_i in the digest is usually carried out after the polyphosphates formed are hydrolyzed to P_i by heating the incinerated digest with water for a few minutes.

1) R.J.L. Allen, *Biochem. J.*, **34**, 858 (1940).
2) M. Nakamura, *Nippon Nôgei-kagaku Kaishi*, **24**, 1 (1950).

Then, the question arises: How much of P_i will condense to polyphosphates?; and how long would heating in a boiling water bath be necessary to effect complete hydrolysis of polyphosphates, if there is any formation of polyphosphates, and consequently, to obtain a correct value for total phosphorus?

Besides, it sometimes happens that an unusually small value is obtained in the determination of total phosphorus. There are two possibilities to account for this phenomenon: one is experimental error such as incomplete combustion or spilling of a part of the digest in abrupt boiling; the other is the possible vaporization of phosphoric acid during the incineration[3].

This paper deals with the questions raised above.

EXPERIMENTAL

Materials

α-D-Glucose-1-phosphate (G-1-P): Crystalline potassium salt of G-1-P was prepared as reported in the preceding paper[4].

Potato extract: Peeled potatoes were crushed by a porcelain grinder and the pressed juice was centrifuged at 3,000 r.p.m. for 7 min. The supernatant fluid was used.

Radioactive phosphoric acid ($H_3P^{32}O_4$): This was obtained from the Japan Radioisotope Association.

Solutions

See the preceding paper[4].

Determination of radioactivity

An aliquot of a solution, the radioactivity of which is to be determined, was placed in a Lauritzen plate or in a small glass plate and dried under an infrared lamp with precaution. The radioactivity was determined with a Geiger-Müller counter attached to an Aloka decimal scaler.

Procedures

A. Methods employing the colorimetric determination of Inorganic orthophosphate according to Takahashi.

A1. Experiments using orthophosphate and routine procedures: One ml of the standard phosphate solution (containing 84.0 mg of KH_2PO_4/100 ml) and 1.5 ml of 15% sulfuric acid or 1.2 ml of 60% perchloric acid were pipetted into a small Kjeldahl flask which had an etched mark-line at 10 ml around the neck. The flask was then heated on a small flame to evaporate off the water until white fumes of acid could be observed. Heating was regulated so as not to drive out the white vapor from the flask, and was continued for an hour. The flask was then cooled and water was added to make up the content to 10 ml. The flask was heated in a vigorously boiling water bath and 2 ml aliquots were withdrawn before and after heating for 5, 10, and 15 min., respectively, for the determination of P_i according to the method of Takahashi[5] as described in the preceding paper.

A2. Long-term heating of orthophosphate in concentrated sulfuric acid: A small amount of potassium dihydrogen phosphate (approximately 45 mg) was heated with 10 ml of concentrated sulfuric acid in a Kjeldahl flask for about 3 hours, the temperature being kept at the boiling point of sulfuric acid. After cooling, a 0.2 ml aliquot was pipetted into a 10 ml volumetric flask and water was added up to the mark. The flask was heated in a vigorously boiling water bath, and 2 ml aliquots were withdrawn before and after heating for 5, 10, and 15 min., respectively, for the determination of P_i by the method of Takahashi.

A3. Experiments with samples containing organic phosphorus: The procedure was the same as described in A1, except that the following samples were used as the source of organic phosphorus:

a) A solution of G-1-P (232.0 mg/100 ml)
b) Potato extract.

B. Method employing radioactive phosphorus.

Both incineration and hydrolysis procedures were the same as described in A1, except that radioactive orthophosphoric acid ($H_3P^{32}O_4$) was added to the standard phosphate solution. The mouth of the flask was covered with a piece of filter paper in order to catch the phosphoric acid vapor escaping from the flask.

Before and after heating the diluted digest in a boiling water bath in the same way as described in A1, 2 ml aliquots were withdrawn and P_i was extracted with 4 ml of isobutanol in the form of phosphomolybdic acid[6]. One-ml aliquot of the isobutanol layer was used for the determination of radioactivity. The radioactivity of 1 ml aliquot of the aqueous layer was also determined to examine the efficiency of extraction.

RESULTS AND DISCUSSION

The results, summarized in Table I, show

3) M. Furukawa, M. Oida, Y. Nakamura, S. Kasuga and H. Yoshikawa, *Seikagaku*, **24**, 76 (1952).
4) K. Mori and M. Nakamura, This Bulletin, **23**, 272 (1959).
5) H. Takahashi, *Seikagaku*, **26**, 690 (1955).
6) Y. Yoshizawa, *Seikagaku*, **24**, 219 (1953).

TABLE I. POLYMERIZATION OF ORTHOPHOSPHATE IN THE WET ACID DIGESTION AND HYDROLYSIS OF POLYMERIZED ORTHOPHOSPHATE DURING HEATING THE DIGEST WITH WATER

For experimental procedures, see the text. Figures in the table represent: under method **A**, optical densities read in a Hitachi model EPO-A photoelectric colorimeter fitted with filter R (maximum absorption at 660 mμ) and a cuvette with 1-cm light path; under method **B**, counts per minutes.

Acid used for the incineration	Sample	Method of determination	Time of hydrolysis (min.) 0	5	10	15	Control
Sulfuric acid	KH_2PO_4	A.1.[a]	.331	.330	.324	.329	.329[b]
	KH_2PO_4	A.2.[a]	.411	.409	.407	.409	
	G-1-P	A.3.	.328	.326	.329	.334	.332[c]
	Potato extract	A.3.	.343	.346	.343	.341	
	$H_3P^{32}O_4$	B.[a][d]	3848[e]	3912	3939	3941	3934[b]
Perchloric acid	KH_2PO_4	A.1.[a]	.351	.354	.355	.350	.349[b]
	G-1-P	A.3.	.333	.327	.335	.331	.332[c]
	Potato extract	A.3.	.346	.349	.343	.348	
	$H_3P^{32}O_4$	B.[a][d]	3697[f]	3785	3764	3893	3803[b]

a) Average value of four experiments.
b) Determined without heating with acid.
c) Determined after hydrolysis in 1 N sulfuric acid for 7 min. in a vigorously boiling water bath.
d) With a deviation of ±60.
e) One ml of the aqueous layer had radioactivity of 32 c.p.m.
f) One ml of the aqueous layer had radioactivity of 38 c.p.m.

that there occurs only a very slight, if any, condensation of orthophosphate to polyphosphates under the conditions employed in these experiments.

Roux et al.[7] showed that condensation of orthophosphate to polyphosphates occurred when the former was heated for about 10 min. at 350°. As the boiling point of sulfuric acid (98.3%) is 338° and that of perchloric acid 203°, formation of polyphosphates is likely to occur in sulfuric acid incineration. Actually, however, in our experiments polyphosphates were not formed from orthophosphate. That the concentration of orthophosphate was very low might perhaps be responsible for the failure to find polymerized phosphates, or the heat, which was evolved when the digest was diluted with water, might be sufficient to hydrolyze all the polymerized phosphates.

7) H. Roux, E. Thilo, H. Grunze and M. Viscontini, *Helv. Chim. Acta*, **38**, 15 (1955).

When heating was carried out with precaution not to drive out the white vapor from the flask, there was no loss of phosphoric acid. For instance,

> Radioactivity adhered to the filter paper placed at the mouth of the flask/total radioactivity added to the flask=24/20,000≒0.1%.

As the boiling point of orthophosphoric acid is 300.1°, it will vaporize at the boiling point of sulfuric acid. Thus, the loss of phosphoric acid will result if the white vapor is allowed to escape from the flask.

As can be seen from the table, extraction with isobutanol was effective in removing more than 98% of phosphomolybdic acid from the aqueous layer.

Acknowledgment The authors wish to express their sincere thanks to Prof. S. Funahashi for his interest in this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 4, p. 281~288, 1959]

Studies on the Decomposition of Nucleic Acid by Microorganisms

Part VI. On the Ribosidase of *Aspergillus oryzae* Acting on 6-Hydroxypurine Ribonucleosides and their 5′-Monophosphates*

By Akira KUNINAKA

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Received September 26, 1958

An enzyme preparation has been purified about forty fold from a strain of *Aspergillus oryzae*. This preparation catalyzes the hydrolytic cleavage of inosine, guanosine, 5′-IMP, and 5′-GMP to the corresponding bases (hypoxanthine and guanine) and ribose or its 5-phosphate. No change is observed in the relative rates of hydrolysis of these four substrates with purification, indicating that a single enzyme "ribosidase", which cleaves specifically the ribosidic linkages of 6-hydroxypurine ribonucleosides (inosine and guanosine) or their 5′-phosphates, does exist in *Aspergillus oryzae*. It has been found that 5′-AMP, 5′-CMP, 5′-UMP, adenosine, cytidine, uridine, and various 3′- or 2′-mononucleotides are not cleaved by the enzyme. No evidence for the requirement of ATP, P, or PP has been found. This enzyme is rather thermostable in the pH range from 5.0 to 6.5. The optimum conditions for activity are at about 45°C and pH 4.0.

According to the prevailing theory[1], the phosphoric acid group in the nucleotides has to be removed before the ribosidic linkage can be resolved by enzymes. Ishikawa and Komita[2] discovered that dog pancreas was capable of discrupting the ribosidic linkages of guanylic and xanthylic acids without previous cleavage of the phosphate. Moreover, Komita[3,4] has separated this enzyme from purine nucleosidase, and named it nucleotide-N-ribosidase. However, Schlenk[5] indicated another possible interpretation of these experiments in the light of more recent observations. Thus, proof of the existence of nucleotide-N-ribosidase was lacking.

Recently, the author[6,7,8] has furnished the first evidence for the conclusion that hydrolytic enzyme for the cleavage of the bond between hypoxanthine and R5P does exist in *Aspergillus oryzae*. A little later, Hurwitz et al.[9] have also independently demonstrated the enzymic cleavage of ribosidic linkage in 5′-AMP using *Azotobacter vinelandii*. In the present paper, it is indicated that the ribosidase of *Aspergillus oryzae* acts not only on 5′-IMP but also on inosine, 5′-GMP, and guanosine.

METHODS

The various nucleosides, nucleoside-3′-phosphates, 5′-AMP and ATP used in this study were commercial preparations. Preparations of the nucleoside-3′-phosphates contained the corresponding 2′-isomers together, and were used without the removal of them. 5′-IMP was prepared by the method of Marmur et al.[10]. The

* Presented at the meeting of the Agricultural Chemical Society of Japan, Kyoto, May 3, 1958.

The following abbreviations are used here: for monophosphates of adenosine, inosine, guanosine, uridine, and cytidine,—AMP, IMP, GMP, UMP, and CMP; yeast ribonucleic acid,—RNA; adenosine-5′-triphosphate,—ATP; inorganic orthophosphate,—P; inorganic pyrophosphate,—PP; ribose-5-phosphate,—R5P.

1) P. A. Levene and A. Dmochowski, *J. Biol. Chem.*, **93**, 563 (1931).
2) H. Ishikawa and Y. Komita, *J. Biochem.*, **23**, 351 (1936).
3) Y. Komita, *J. Biochem.*, **25**, 405 (1937).
4) Y. Komita, *J. Biochem.*, **27**, 23 (1938).
5) F. Schlenk, The Nucleic Acids (ed. E. Chargaff, J. N. Davidson), Academic Press Inc., New York, 1955, Vol. 2, p. 329.
6) A. Kuninaka, *J. Agr. Chem. Soc. Japan*, **30**, 583 (1956).
7) A. Kuninaka, *Koso Kakagu Shinpojiumu*, **12**, 65 (1957).
8) A. Kuninaka, *J. Gen. Appl. Microbiol.*, **3**, 55 (1957).
9) J. Hurwitz, L.A. Heppel and B. L. Horecker, *J. Biol. Chem.*, **226**, 525 (1957).
10) J. Marmur, F. Schlenk and R. N. Overland, *Arch. Biochem. Biophys.*, **34**, 209 (1951).

other nucleoside-5′-phosphates were prepared enzymatically from RNA. The detailed procedure will be published separately.

Inorganic and organic phosphates were determined according to the Fiske-Subbarow method[11]. The colorimetric method of Nelson[12] to observe the appearance of free ribose or ribose phosphate was employed, except that the treatments with barium hydroxide and zinc sulfate were omitted. Protein was determined by the method of Lowry et al.[13].

As the main source of ribosidase, culture filtrates of *Aspergillus oryzae* var. No. 13, a strain employed in soy-manufacture[14], were used. In usual cases the strain was grown at 30°C for ten days on surface of a medium having the following composition: glucose, 5%; polypeptone, 0.5%; monobasic potassium phosphate, 0.05%; dibasic potassium phosphate, 0.05%; calcium chloride, 0.04%; magnesium sulfate, 0.04%. Details concerning the procedure for each enzyme preparation will be described under the paragraph "RESULTS". Zone electrophoresis was carried out using potato starch as the supporting material. Calcium phosphate gel was prepared by the method of Keilin and Hartree[15].

The assay procedure of the enzyme (ribosidase) was based on the appearance of reducing sugar*. The following assay mixture was used: 0.1 ml of 25 mM substrate, 0.1 ml of 1 M sodium fluoride, 0.5 ml of 0.1 M acetate buffer, pH 4.0, 0.25 to 2.0 units of enzyme, and water to give a volume of 1.0 ml. After thirty minutes at 45°C, reducing sugar (ribose or R5P) was measured. A unit of enzyme activity was defined as equal to the amount required for the formation of 1 μmol. of reducing sugar or purine base per hour at 45°C under the above conditions. In this assay, controls lacking enzyme or substrate were run. All enzyme reactions were performed under an atmosphere of air. The above conditions (composition of reaction mixture, pH, temperature, and time) were employed throughout all enzymic reactions described in this paper, except when otherwise indicated. Under these conditions, the reaction rate was found to be linear with time and directly proportional to enzyme concentration. In case of using amounts of the enzyme larger than 2.0 units, proportionality between the rate and the enzyme concentration was preserved in a somewhat wider range for inosine than for 5′-IMP.

RESULTS

I. The Relation between 5′-IMP-N-Ribosidase Activity and Inosine Ribosidase Activity. In the preceding papers[6,7,8,16] it was shown that *Aspergillus oryzae* contained ribosidase (purine ribonucleoside hydrolase) acting on inosine and guanosine, and 5′-IMP-N-ribosidase acting on the ribosidic linkage of 5′-IMP. To begin with, the relation between 5′-IMP-N-ribosidase activity and inosine ribosidase activity was investigated by the following experiments:

(1) **Influence of culture conditions** 5′-IMP-N-ribosidase and inosine ribosidase activities in culture filtrates were measured during growth. Both activities seemed to appear simultaneously in the medium at approximately the same ratio* (Fig. 1).

(2) **Solubility in acetone** The fraction soluble in 20% and insoluble in 40% acetone at pH 6.5 contained enzymes having the majority of both original activities. (In this experiment, the fraction precipitated between 0.6 and 1.0 saturated ammonium sulfate was used as the original enzyme solution.) Treatments with methanol, ethanol, and ammonium sulfate at various pH values also, could not separate both activities.

(3) **Treatments with adsorbents** Both activities were adsorbed onto alumina, obtained from Wako Junyaku Co., 200 mesh, most effectively at about pH 5.0, and eluted together almost perfectly by 0.1 M dibasic sodium phosphate solution. For the elution of both activities, 1 M dibasic sodium phosphate solution, which was

11) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).
12) N. Nelson, *J. Biol. Chem.*, **153**, 375 (1944).
13) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
14) M. Kibi, *J. Agr. Chem. Soc. Japan*, **24**, 278 (1951).
15) D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (*London*), **B 124**, 397 (1938). Cited by S. P. Colowick "Methods in Enzymology" Academic Press Inc., New York, 1955, Vol. 1, p. 98.
* It was preliminarily confirmed that reducing sugar and purine base are formed simultaneously. When necessary, the purine base formed was determined spectrophotometrically after separation on paper by means of either chromatography or electrophoresis[6,7,8,16].
16) A. Kuninaka, *J. Agr. Chem. Soc. Japan*, **29**, 801 (1955).
* In the early crude preparation, such as culture filtrates, the ratio of inosine ribosidase to 5′-IMP-N-ribosidase usually ranged from 1.4 to 2.5. However in a simple purification procedure, such as dialysis or treatment with alumina, this ratio was readily reduced to about 1.0. In the course of further purification, no change in the ratio was observed. The presence of the small molecular factor(s) affecting the enzyme reactions in culture filtrates may be suggested. This point is under investigation.

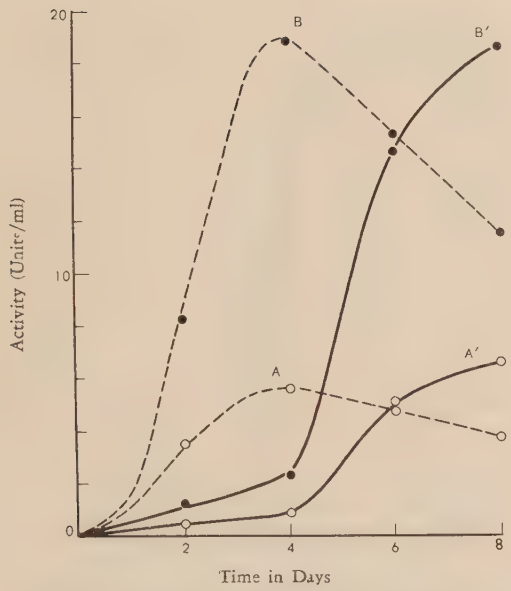


FIG. 1. Activity of Ribosidase in Culture Filtrates of *Aspergillus oryzae* at Various Periods.

Aspergillus oryzae var. No. 13 was grown in 100 ml of the glucose-peptone medium at 30°C for the periods indicated in the Figure. (The ratio of inosine ribosidase activity to 5′-IMP-N-ribosidase activity in each culture filtrate employed in this experiment seemed to be relatively high.)

Curve A: 5′-IMP-N-ribosidase activity (activity of R5P formation from 5′-IMP) in shaking culture filtrates.

Curve A′: 5′-IMP-N-ribosidase activity in surface culture filtrates.

Curve B: Inosine ribosidase activity (activity of ribose formation from inosine) in shaking culture filtrates.

Curve B′: Inosine ribosidase activity in surface culture filtrates.

much more effective than 0.1 M solution for elution of most of the other proteins adsorbed from culture filtrates, proved to be much less effective than 0.1 M solution. A 1 M solution of sodium bicarbonate and a 0.3 to 0.6 saturated solution of ammonium sulfate were also found effective for elution of both activities, while 0.1 M solution of sodium pyrophosphate or sodium perborate was much less effective. Both activities adsorbed onto calcium phosphate gel were eluted together with essentially the same recovery by respective eluants as described in Table I. The effect of various eluants on elution of both activities was rather different from the effect on elution of the total protein

TABLE I. ELUTION OF RIBOSIDASE FROM CALCIUM PHOSPHATE GEL

Eluants	Recovery (%) in eluates		
	Total protein	Activity against 5′-IMP	Activity against Inosine
1M Na_2HPO_4	2.1	14.6	12.0
0.1M Na_2HPO_4	1.6	3.9	3.4
80% glycerol	1.1	2.4	2.8
0.3 saturated $(NH_4)_2SO_4$	0.5	17.0	14.5
1M Na_2HCO_3	1.3	0	0
0.05M Borate buffer, pH 10	0.5	0	0

20 ml of partially purified enzyme solution, the fraction precipitated between 0.6 and 1.0 saturated ammonium sulfate containing 47.2 mg of total protein and 830 units of 5′-IMP-N-ribosidase or 886 units of inosine ribosidase, were treated with 12.5 g of calcium phosphate gel at pH 5.4. The both activities were adsorbed perfectly on to the gel. Elution was carried out by 4 ml of each eluant indicated in the Table from each 2 g of the gel. Recovery of total protein and ribosidase was determined.

adsorbed from culture filtrates. This was also true for adsorption onto corn starch under various conditions. For example, 40% ammonium sulfate solution seemed to prevent specifical adsorption of both activities onto corn starch. Both activities were also not separated by use of a cation exchanger, Amberlite IRC-50, under various conditions.

(4) Action of various drugs To each 5 ml of the enzyme solution, concentrated culture filtrates containing 27.4 units of 5′-IMP-N-ribosidase per ml, 2 g of lead acetate, 2 g of urea, 0.1 g of iodine, 1 ml of water-saturated phenol and 0.1 g of benzalkonium chloride were added. After being allowed to stand at 45°C for two hours, each supernatant solution was dialyzed, and then 5′-IMP-N-ribosidase and inosine ribosidase activities were measured. Both activities were not recognized in each solution. This apparently indicates that these drugs perfectly inactivate both 5′-IMP-N-ribosidase and inosine ribosidase. On the other hand, inactivation by 0.1 g of mercuric chloride was comparatively mild under the same conditions. Thus, both enzyme activities per protein were able to be raised up to three fold by treatment with mercuric chloride, although about 70% of the total activities was lost. The splitting rates of 5′-IMP and inosine were not influenced signi-

ficantly by the addition of 0.01 M (final concentration) of potassium cyanide, monoiodoacetic acid, or oxalic acid to the reaction mixture.

(5) Influence of temperature and pH Both 5′-IMP-N-ribosidase and inosine ribosidase were rather thermostable from pH 5.5 to pH 6.0 (Fig. 2). After heating the enzyme solotion

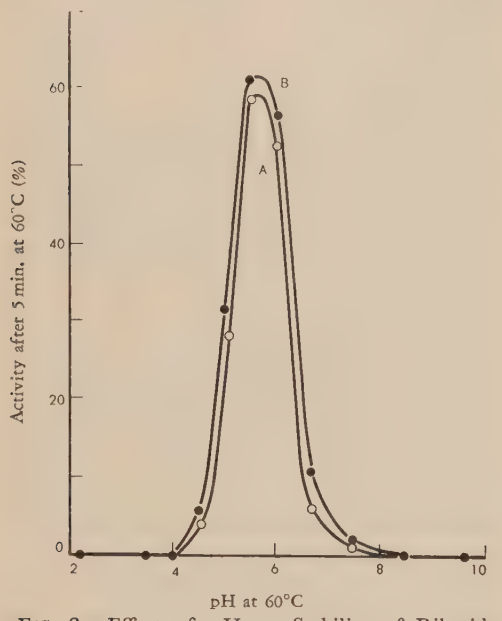


FIG. 2. Effect of pH on Stability of Ribosidase at 60°C.

The enzyme solution corresponding to "ethanol fraction" (described in "RESULTS II") was employed.
Curve A: 5′-IMP-N-ribosidase activity.
Curve B: Inosine ribosidase activity.

("first zone fraction" described in "RESULTS II") for 10 min., 20 min., 30 min., 40 min., and 60 min. at 55°C, pH 5.5, 80%, 70%, 58%, 50%, and 32% of both activities were retained respectively. Furthermore, no difference was observed between stability of 5′-IMP-N-ribosidase against various temperatures and that of inosine ribosidase (Fig. 3). The optimum conditions for the activities of 5′-IMP-N-ribosidase and inosine ribosidase were found to be at about 45°C and pH 4.0. The results are given in Figs. 4 & 5.

(6) Action of ribosidase on mixed substrates The results described above suggest that two

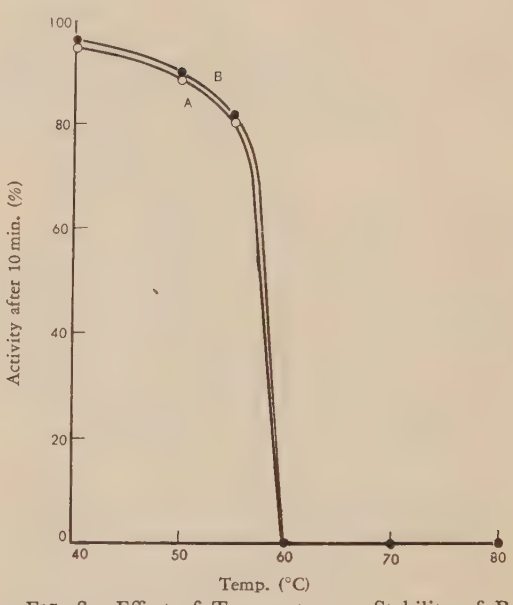


FIG. 3. Effect of Temperature on Stability of Ribosidase at pH 5.5.

"Acetone fraction" diluted 10-fold (described in "RESULTS II") was used as the enzyme solution.
Curve A: 5′-IMP-N-ribosidase activity.
Curve B: Inosine ribosidase activity.

enzyme activities are associated with the same protein. The same results were also obtained when the enzyme acted on mixtures of the two substrates. The data given in Table II may be assumed to indicate that both 5′-IMP and inosine are attacked by the same enzyme.

(7) Zone electrophoresis Additional evidence for the association of the two activities results from their behaviour on zone electrophoresis. When electrophoresis was carried out using starch as a supporting material in a trench of $46 \times 2.5 \times 1.0$ cm, both activities migrated equally towards the cathode or the anode at various pH values between 4.5 and 9.0. Fig. 6 shows typical results obtained at pH 4.54 and pH 8.62. No separation of the two enzyme activities was observed, nor was either activity resolved into the two fractions.

This series of experiments may be considered to indicate that a single enzyme ribosidase catalyzes the hydrolytic cleavage of the ribosidic linkages in both 5′-IMP and inosine. From

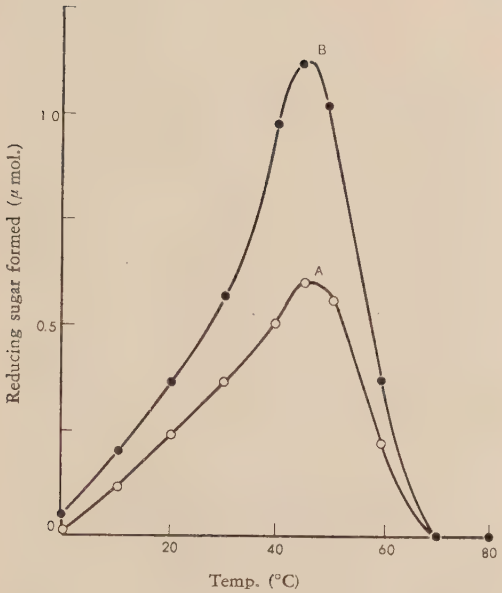


FIG. 4. Activity of Ribosidase at Various Temperatures.

Culture filtrates were employed after mild dialysis as the enzyme solution.
Curve A: 5′-IMP-N-ribosidase activity.
Curve B: Inosine ribosidase activity.

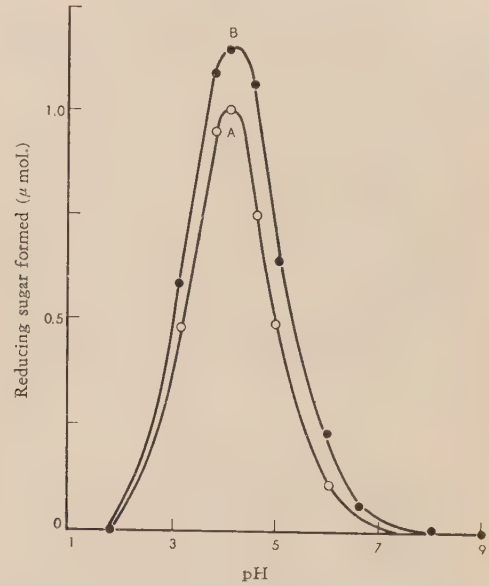


FIG. 5. Activity of Ribosidase at Various pH Values.

The enzyme solution corresponding to "ethanol fraction" was used.
Curve A: 5′-IMP-N-ribosidase activity.
Curve B: Inosine ribosidase activity.

TABLE II. ACTION OF *ASPERGILLUS* RIBOSIDASE ON MIXED SUBSTRATES

Time (min.)	Reducing sugar liberated (μ mol./ml) From 5′-IMP 2.5 μ mol./ml	From 5′-IMP 5.0 μ mol./ml	From inosine 2.5 μ mol./ml	From inosine 5.0 μ mol./ml	From 5′-IMP (2.5 μ mol./ml) and inosine (2.5 μ mol./ml)
15	0.380	0.470	0.320	0.330	0.330
30	0.655	0.830	0.630	0.705	0.710
45	0.800	1.060	0.840	1.020	1.000
60	0.950	1.350	1.100	1.335	1.250

The enzyme solution corresponding to "acetone fraction" diluted 10 fold was used.

this point, the action of several molds on the ribosidic lihkage of 5′-IMP or inosine was studied. Culture filtrates of *Aspergillus oryzae* A*, *Aspergillus oryzae* S-4-15*, *Aspergillus niger* a*, and *Penicillium multicolor* G.M. and P*, as well as *Aspergillus oryzae* var. No. 13 were observed to be capable of splitting the ribosidic linkages of both 5′-IMP and inosine at pH 5.0 and 45°C. As a contrast, those of *Rhizopus nigricans* NRRL No. 45*, *Rhizopus delmer* var. mininus R-1-4*, *Mucor spinescens* Mu-3*, *Monascus purpureus* 3-1*, *Penicillium citrinum* Thom 1131*, *Penicillium spinulosum* Thom*, *Penicillium expansum* 1327*, and *Aspergillus fumigatus* 26-12* were observed to be incapable of appreciably splitting the ribosidic linkages of both 5′-IMP and inosine at least under the present conditions. It thus seems probable that

* I wish to express my thanks to Assist. Prof, H. Iizuka, the Institute of Applied Microbiology, University of Tokyo, for kindly supplying the strains used in this study.

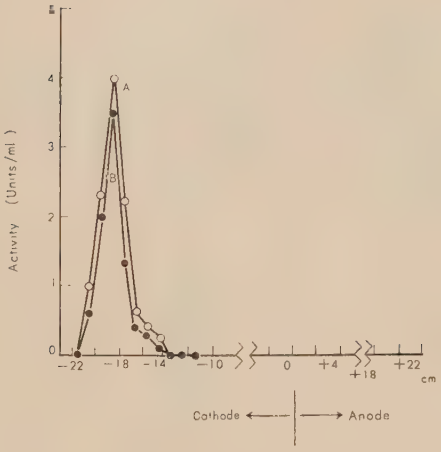


FIG. 6a. Zone Electrophoresis in 0.05 M Acetate Buffer, pH 4.54, with 400 V and 10 to 11.8 mamp for 14.5 hr.

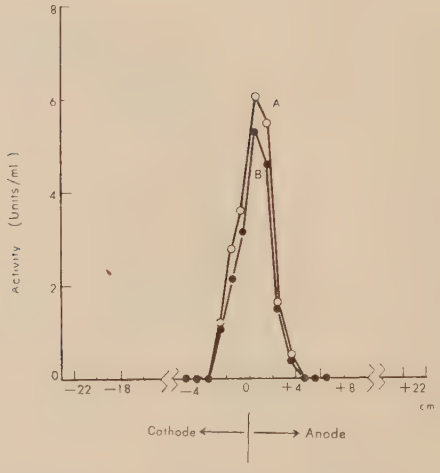


FIG. 6b. Zone Electrophoresis in 0.1 M Veronal-Hydrochloric Acid Buffer, pH 8.62, with 200 V and 10 to 11 mamp for 15.0 hr.

One ml of the partially purified enzyme solution corresponding to "ethanol fraction" containing about 70 to 100 units was put on the band of 1 cm wide of the starch column. After running each band of 1 cm wide was eluted with 5 ml of distilled water. The supernatant fluid was used for measurement of activity.

Curve A: 5′-IMP-N-ribosidase activity.
Curve B: Inosine ribosidase activity.

the enzyme "ribosidase" acting on both 5′-IMP and inosine is distributed among various fungi, especially, among the strains which belong to the *Aspergillus* genus.

The Km values for 5′-IMP and inosine calculated were found to be approximately 5.46×10^{-3} M and 2.63×10^{-3} M, respectively (Fig. 7). Thus, the affinity of ribosidase with inosine may be closer than that with 5′-IMP.

In contrast with AMP-ribosidase of *Azotobacter vinelandii*[9], it is apparent from the following experiment that ribosidase of *Aspergillus oryzae* is not activated by ATP. The partially purified enzyme preparation, corresponding to the "ethanol fraction", was incubated with 2.5 μmol. of 5′-IMP or inosine in the presence of 2 μmol. of ATP at 45°C and pH 4.0 for 30 minutes (total volume, 1.0 ml). In the absence of ATP, 0.54 μmol. of reducing sugar was formed from 5′-IMP and inosine, respectively. In the presence of ATP, 0.49 and

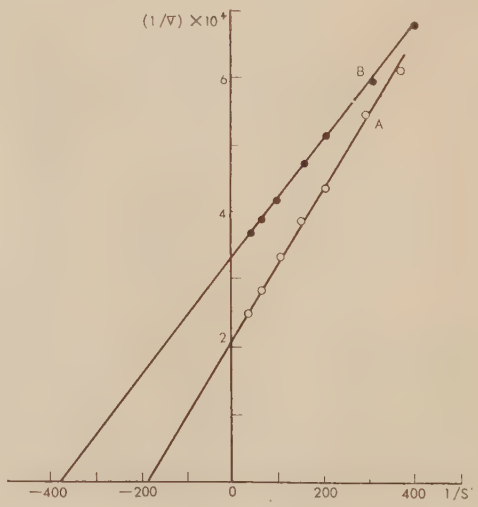


FIG. 7. Determination of the Km Values.

The Km values were determined for 5′-IMP and inosine at 45°C and pH 4.0, using "acetone fraction" diluted 10-fold as the enzyme solution.

Km (5′-IMP) = M/183 = 5.46×10^{-3} M (Curve A)
Km (inosine) = M/380 = 2.63×10^{-3} M (Curve B)

0.51 μmol. of reducing sugar were formed from 5′-IMP and inosine, respectively.

II. Purification and Substrate Specificity of *Aspergillus oryzae* Ribosidase. As described above, *Aspergillus oryzae* seems to contain an enzyme "ribosidase" having both 5′-IMP-N-ribosidase and inosine ribosidase activities. The enzyme was then purified as follows: 4940 ml of the culture filtrate of *Aspergillus oryzae* var. No. 13 was concentrated in vacuo, and dialyzed overnight against running water. To the undialyzable raction (180 ml) four volumes of ethanol were added. The resulting precipitate was separated from the supernatant by centrifugation, and dried up in a desiccator (ethanol fraction, 1.5 g). The "ethanol fraction" was dissolved in 21 ml of distilled water and subjected to zone electrophoresis in a trench of 45×2×5 cm using 0.1 M veronal-hydrochloric acid buffer at pH 9.02 with 150 V and about 23 mamp., for 24 hours. After running, the band containing ribosidase activity (15 cm-wide) was eluted with 407 ml of distilled water (first zone fraction). This fraction was further subjected to second zone electrophoresis in 0.1 M veronal-hydrochloric acid buffer at pH 8.85 with 200 V and 40 mamp. for 20 hours. After running, the band with high ribosidase activity (3 cm-wide) was eluted with 272 ml of distilled water (second zone fraction). This "second zone fraction" was concentrated to 46 ml in vacuo. The precipitate was removed. To the supernatant adjusted to pH 5.5 with acetic acid, a two-third-volume of acetone was added. The precipitate formed was dissolved in distilled water and dialyzed against running water. Fifteen ml of a colorless and clear solution was obtained (acetone-fraction). The above procedure resulted in a purification of approximately forty fold. A summary of the purification procedure is given in Table III. The final preparation was free of phosphomonoesterase activity. As described in Table III, the final preparation was recognized to split 5′-GMP and guanosine, as well as 5′-IMP and inosine. Furthermore, the ratio of the activities of "ethanol fraction" against 5′-IMP, inosine, 5′-GMP, and guanosine was not appreciably altered by further purification. The reducing sugars formed from 5′-GMP and guanosine were identified by the method described previously[6,7,8] as R5P and ribose, respectively. Both P and PP not were recognized to be involved in the enzymic splitting of 5′-GMP and guanosine, as well as in the splitting of 5′-IMP and inosine[8]. It may be concluded that these reactions are not (pyro)phosphorolysis but hydrolysis. Evidence for requirement of ATP was also not recognized in the splitting of 5′-GMP and guanosine. The ribosidic linkages in adenosine, uridine, cytidine, 3′- and 2′-isomers of AMP, GMP, IMP, XMP, CMP,

Table III. Summary of Purification Procedure

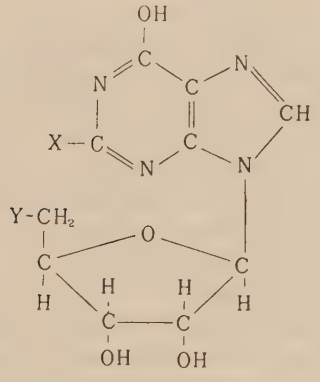
Fraction	Total Protein mg	Specific activity† units per mg protein	Relative activity for 4 substrates			
			5′-IMP	Inosine	5′-GMP	Guanosine
Culture filtrate	1580.8	11.9	100	142		
Ethanol fraction	565.0	16.3	100	107	12	32
First zone fraction	151.4	50.8	100	106	13	30
Second zone fraction	90.3	70.5	100	98	15	27
Acetone fraction	5.7	447.4	100	96	12	27

† Specific activity was determined using 5′-IMP as substrate.

and UMP, as well as 5′-isomers of AMP, CMP, and UMP, were not recognized to be splitted by *Aspergillus oryzae* ribosidase. It is as yet undecided whether ribosidase splits 5′-XMP or xanthosine, although preliminary experiments suggest that both compounds are split by the enzyme at rather small rates.

DISCUSSION

On the basis of the results described in this paper, *Aspergillus oryzae* var. No. 13 has been shown to contain a ribosidase acting on the ribosidic linkages of the compounds with the structure shown in Fig. 8. Conditions necessary



X=H, NH_2, (or OH)
Y=OH, or OPO_3H_2

FIG. 8. The General Structure of the Substrates of *Aspergillus oryzae* Ribosidase.

for the structure of the substrate may be summarized as follows:

(i) The base moiety is to be purine nucleus containing a hydroxy group in the 6 position. (An amino group in the 6 position seems to completely interfere with the enzymic cleavage of ribosidic linkage. Pyrimidine nucleosides or their phosphates are not attacked by the enzyme.)

(ii) Both the 2′ and 3′ positions of ribose moiety are not to be esterified with P.

Although the presence of an amino group in the position 2 in purine moiety seems to somewhat interfere with the cleavage of ribosidic linkage, the influence of the kind of the group in this position on ribosidase action is not recognized to be vital. It is interesting to note that the 5′-phosphate group does not appear to have a remarkable effect on reacton. Further experiments on substrate specificity are now being carried out.

Both ribosidase from *Aspergillus oryzae* and 5′-AMP-ribosidase from *Azotobacter vinelandii*[9] are nonphosphorolytic hydrolases, and act on nucleoside-5′-phosphates but do not act on 2′- or 3′-isomers*. However, these enzymes are distinguishable from each other in regard to either detailed substrate specificity or relation to ATP. The special feature of *Aspergillus* ribosidase may be to hydrolyze both nucleosides and 5′-nucleotides.

There are various hydrolytic or (pyro)phosphorolytic enzymes, acting on the ribosidic linkages in either nucleosides or nucleotides. Therefore, the confirmation of the existence of a particular enzyme, nonphosphorolytic ribosidase acting on both nucleosides and 5′-nucleotides, in *Aspergillus oryzae* may present interesting evidence from the view point of comparative biochemistry. Furthermore, this enzyme may be employed to establish the location of the phosphate group in guanylic or inosinic acid.

Acknowledgements I wish to express my hearty thanks to Emeritus Prof. K. Sakaguchi, University of Tokyo, for his constant guidance and encouragement. I am also indebted to Prof. K. Arima, University of Tokyo, for his valuable advice throughout this work, to Dr. M. Onuki and Mr. M. Kibi, directors of Yamasa Shoyu Co. Ltd., for their valuable suggestions, and to Mr. G. Motoki and Miss K. Suzuki of this laboratory for their assistance in carrying out the experiments.

* It is very interesting to find that both hydrolases and (pyro) phosphorylases, studied so far, act on nucleoside-5′-phosphates but do not act on 2′- or 3′-isomers.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 4, p. 289~310, 1959]

The Structure of an Antibiotic Kanamycin

By Hiroshi Ogawa, Teiichiro Ito, Shinichi Kondo and Shigeharu Inoue

Research Laboratory, Meiji Seika Co., Ltd.

Received October 4, 1958

The antibiotic kanamycin was degraded with methanolic hydrogen chloride and was determined to be composed of three compounds: deoxystreptamine, 6-amino-6-deoxy-D-glucopyranose and 3-amino-3-deoxy-D-glucopyranose. From the chemical and physical data on the antibiotic and its fragments, kanamycin was shown to be O-α-6-amino-6-deoxy-D-glucopyranosyl-(1→4 or 6)-O-[α-3-amino-3-deoxy-D-glucopyranosyl-(1→6 or 4)]-1,3-diamino-1, 2, 3-trideoxy-*myo*-inositol.

Kanamycin[1] found by H. Umezawa et al. is a therapeutically useful antibiotic, produced by *Streptomyces kanamyceticus*[2]. The studies about its chemical structure have been carried out by H. Umezawa et al.[3], M.J. Cron et al.[4], S. Umezawa et al.[5], and in our laboratory[6], and their brief reports have been published. We should now like to describe more detailed and additional experimental data about its chemical studies.

The homogeneity of kanamycin was first determined by paper chromatography[2a,b,4a] and countercurrent distribution, and a tentative empirical formula $C_{18}H_{36}N_4O_{11}$ to this compound was assigned by the molecular weight determination[4a] and the analyses of the crystalline monosulfate, free base, N-acetate, N-benzoate, picrate and several shiff bases[3a,4a]. This formula has been further confirmed by the degradation studies.

Kanamycin free base is soluble in water, slightly soluble in the lower alcohols but insoluble in non-polar organic solvents, and the monosulfate is soluble in water, but insoluble in organic solvents.

Kanamycin showed only weak end-absorption in the ultraviolet specrtrum. The infrared absorption spectra of kanamycin free base and the monosulfate in Nujol are shown in Fig. 1 and 2. These spectra suggested that kanamycin was a polyamino polyhydroxy compound. The intense bands in the 1,000-1,200cm^{-1} region was thought to be typical absorptions of carbohydrate.

The thiosemicarbazone test[7] for the potentially free carbonyl group was negative.

1) T. Takeuchi, T. Hikiji, K. Nitta, S. Yamazaki, S. Abe, H. Takayama and H. Umezawa, *J. Antibiotics*, **A10**, 107 (1957).

2) a. H. Umezawa, M. Ueda, K. Maeda, K. Yagishita, S. Kondo, Y. Okami, R. Utahara, Y. Osato, K. Nitta and T. Takeuchi, *J. Antibiotics*, **A10**, 181 (1957).
b. K. Maeda, M. Ueda, K. Yagishita, S. Kawaji, S. Kondo, M. Murase, T. Takeuchi, Y. Okami and H. Umezawa, *ibid.*, **A10**, 228 (1957).

3) a. K. Maeda, M. Murase, H. Mawatari and H. Umezawa, *J. Antibiotics*, **A11**, 73 (1958).
b. K. Maeda, M. Murase, H. Mawatari and H. Umezawa, *ibid.*, in press (1958).

4) a. M. J. Cron, D. L. Johnson, F. M. Palermiti, Y. Perron, H. D. Taylor, D. F. Whitehead and I. R. Hooper, *J. Am. Chem. Soc.*, **80**, 752 (1958).
b. M. J. Cron, O. B. Fardig, D. L. Johnson, H. Schmitz, D.F. Whitehead, I. R. Hooper and R. U. Lemieux, *ibid.*, **80**, 2342 (1958).
c. H. Schmitz, O. B. Fardig, F. A. O'Herron, M. A. Rousche and I. R. Hooper, *ibid.*, **80**, 2912 (1958).
d. M. J. Cron, O. B. Fardig, D. L. Johnson, D. E. Whitehead, I. R. Hooper and R. U. Lemieux, *ibid.*, in press (1958).
e. M. J. Cron, D. I. Evans, F. M. Paierniti, D. E. Whitehead, I. R. Hooper, Paul Chu and R. U. Lemieux, *ibid.*, in press (1958).

5) a. S. Umezawa, Y. Ito and S. Fukatsu, *J. Antibiotics*, **A11**, 120 (1958).
b. S. Umezawa, Y. Ito and S. Fukatsu, *ibid.*, in press (1958).

6) a. H. Ogawa and T. Ito, *J. Antibiotics* **A10**, 267 (1957).
b. H. Ogawa, T. Ito, S. Inoue and S. Kondo, *ibid.*, **A11**, 70 (1958).
c. H. Ogawa, T. Ito, S. Inoue and S. Kondo, *ibid.*, **A11**, 72 (1958).
d. H. Ogawa, T. Ito, S. Inoue and S. Kond, *ibid.*, in press (1958).
e. H. Ogawa, T. Ito, S. Kondo and S. Inoue, *ibid.*, in press (1958).

7) Some of the potentially free carbonyl group showed no absorption bands of typical C=O groups in the ultraviolet and infrared region; for example the aldehyde group of streptomycin was only indicated by absorption maxima at 270 mμ by its thiosemicarbazone.
a. L. K. Evans and A. E. Gillam, *J. Chem. Soc.*, **1943**, 565.
b. R. Donovick, G. Rake and J. Fried, *J. Biol. Chem.*, **164**, 173 (1946).
c. Q. R. Bartz, J. Corntroulis, H. M. Crooks, Jr., and M.C. Rebstock, *J. Am. Chem. Soc.*, **68**, 2164 (1946).

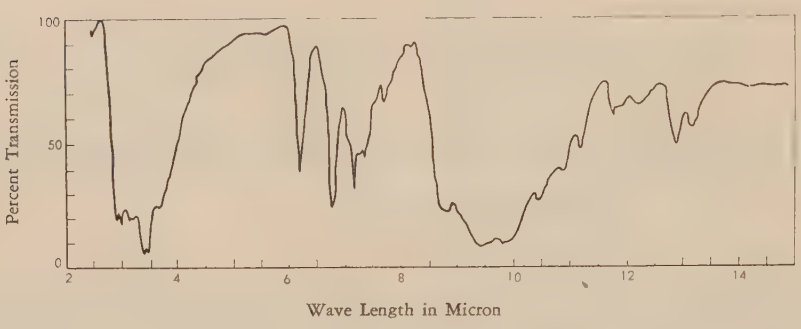


FIG. 1. Infrared Spectrum of Kanamycin Base in Nujol.

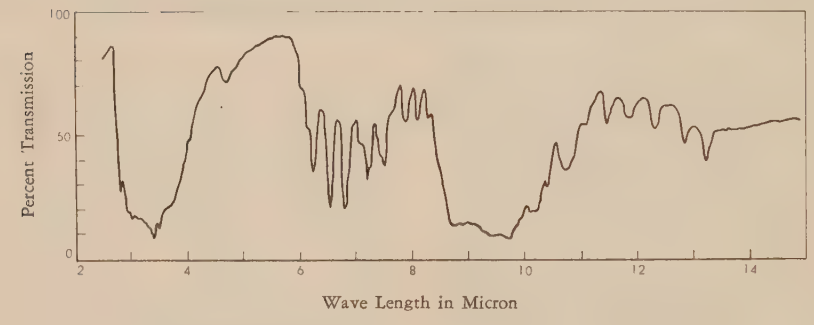


FIG. 2. Infrared Spectrum of Kanamycin Monosulfate in Nujol.

Kanamycin gave positive ninhydrin, Molisch and Elson-Morgan tests, but negative Sakaguchi and reducing sugar tests. On treatment with 40% sulfuric acid for 1.5 hours at 100°, kanamycin gave a product with the ultraviolet spectrum, of which maxima were at 235 and 278mμ (Fig. 3) and were almost identical to those of furfural. But, this hydrolyzates of kanamycin gave negative aniline acetate test, whereas furfural gave red color with that reagent.

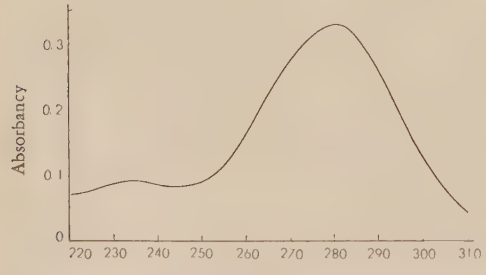


FIG. 3. Ultraviolet Absorption Spectrum of Hydrolysis Product of Kanamycin

(0.5 ml of kanamycin monosulfate soln. (1mg/ml) +4.5ml of 40% H_2SO_4, in boiling water bath for 1.5 hr)

Functional group analyses on kanamycin indicated the absence of methoxyl and C-methyl groups. No hydrogen uptake was observed when an aqueous solution of kanamycin was treated with platinum oxide and hydrogen. The Van Slyke nitrous acid determination indicated that all nitrogen atoms in kanamycin existed as primary amino groups. The potentiometric titration of kanamycin base in water showed that its neutral equivalent was 120.

DEGRADATION STUDIES

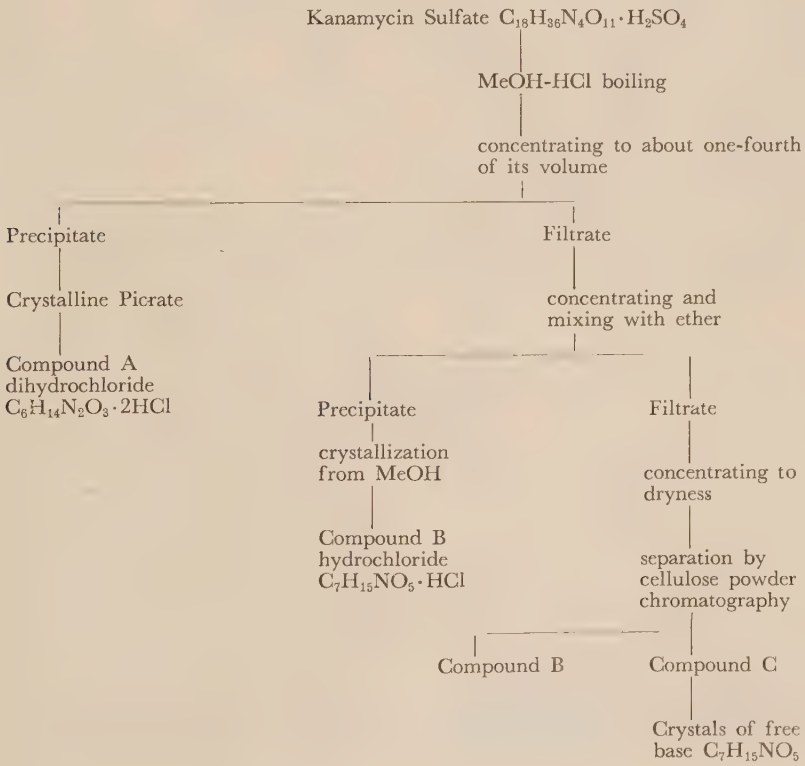
Kanamycin is stable in the alkaline aqueous solution and is more stable than neomycins B and C in the acidic solution. Kanamycin lost almost its activity by refluxing in 6N hydrochloric acid at 100° for about 1 hour. During its degradation in the aqueous media, the solu-

tion was strongly colored and maney degradation products were observed by the paper chromatography, so the acid methanolysis was adopted. But kanamycin was rather resistant to acid methanolysis, so the methanol saturated with hydrogen chloride was used.

The methanolysis products of kanamycin showed three ninhydrin positive spots whose R_F values were 0.28, 0.34 and 0.43 on the paper chromatogram (Tōyō No. 50 filter paper) with the solvent system of *n*-butanol, acetic acid and water (4:1:2.5V.). Those three compounds were named compound A, B and C, respectively. The separation of them was carried out as follows.

Kanamycin was degraded by refluxing a suspension of the monosulfate in methanol saturated with hydrogen chloride. After its activity was almost lost, the solution was concentrated in vacuo to about one-fourth of its volume, yielding amorphous precipitate. Paper chromatography showed that this precipitate contained compound A. This substance was purified as the picrate, and from it, crystalline compound A dihydrochloride was obtained.

The mother liquor, which was separated from the amorphous precipitate of compound A, was further concentrated and was mixed with ethyl ether, resulting precipitate. By paper chromatography, this precipitate was shown to be the mixture of compound B and a small amount of compound C, and the compound B was crystallized as its hydrochloride from methanol. After concentrating the mother liquor, from which compound A and most of compound B were removed, the residue was purified by cellulose powder chromatography. And from the purified fractions, compound C was obtained as its crystalline free base. The following chart summerizes the isolation experiments of three degradation products.



Compound A (2-Deoxystreptamine)

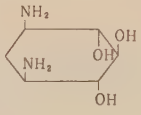
Compound A was characterized as the crystalline dihydrochloride, monohydrochloride, pentaacetate and N,N′-dibenzoate. Analytical data on all of these compounds were in good agreement with the molecular formula, $C_6H_{14}N_2O_3$, for the free base.

Compound A contained two primary amino groups, and the presence of -OH and $-NH_2$ groups was indicated by the infrared absorption (Fig. 4) and by the formation of pentaacetate. Therefore, all oxygen atoms in compound A appeared to exist as hydroxyl. Compound A had neither optical activity nor reducing power and exhibited no ultraviolet absorption.

Compound A dihydrochloride consumed four moles of periodate, whereas its N,N′-dibenzoyl derivative consumed two moles. From this fact, this base must be diaminotrihydroxycyclohexane, and the structure 1,3-diamino-4,5,6-trihydroxycyclohexane is suggested. In 1951, K. Folkers and coworkers[8] reported the isolation of 1,3-diamino-4,5,6-trihydroxycyclohexane (2-deoxystreptamine) by the degradation of neomycin A. As shown in Fig. 4 and 5, the infrared spectrum of compound A dihydrochloride was quite identical with that of deoxystreptamine dihydrochloride from neomycin A, kindly sent to us by Dr. M. Tishler.

K. Folkers et al. reported that deoxystreptamine was meso form and had cis amino groups. J.R. Dyer[9] reported that the hydroxyl and amino groups in deoxystreptamine were all in trans configuration.

So, compound A must have the structure I.



I

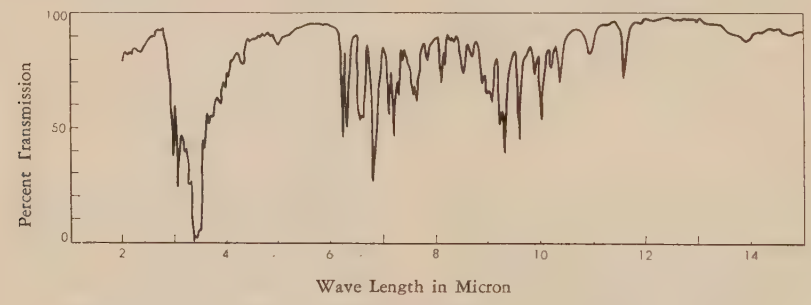


FIG. 4. Infrared Spectrum of Compound A Dihydrochloride in Nujol.

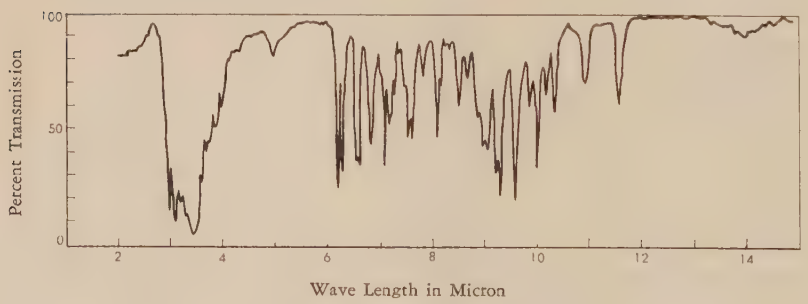


FIG. 5. Infrared Spectrum of Deoxystreptamine Dihydrochloride (from Neomycin A) in Nujol.

8) D. A. Kuehl, Jr., M. N. Bishop and K. Folkers, *J. Am. Chem. Soc.*, **73**, 881 (1951).
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Compound B (Methyl-6-amino-6-deoxy-α-D-glucopyranoside)

Compound B crystallized as the hydrochloride, gave m.p. 188°, $[\alpha]_D^{30}$+133° (in water). Its formulation as $C_7H_{15}NO_5 \cdot HCl$ (m.w. 229.7) was compatible with elemental analyses and a molecular weight of 220, estimated by potentiometric titration. This compound showed pKa′ 8.9, and one methoxyl and one primary amino group, by the group analyses, and had no reducing power and exhibited no ultraviolet absorption. Its infrared absorption spectrum is shown in Fig. 6.

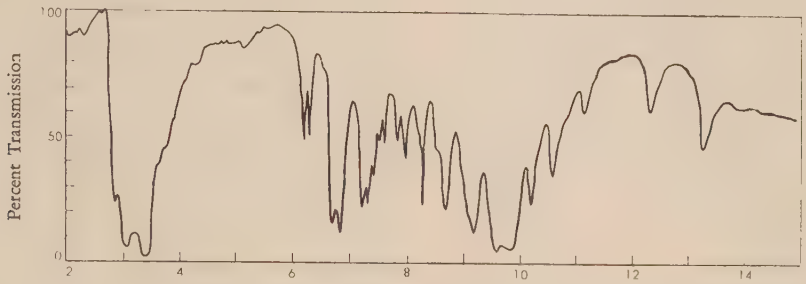


FIG. 6. Infrared Spectrum of Compound B Hydrochloride in Nujol.

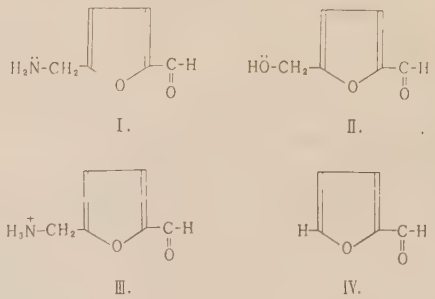
Compound B hydrochloride and its N-acetyl derivative was oxidized readily by periodate, and in the buffer of pH 4.7 both compounds consumed two moles of periodate within about five hours and without formaldehyde formation. This results indicated the presence of three hydroxyl groups on adjacent carbon atoms.

Demethylation of compound B was easily attained by refluxing it with 1N hydrochloric acid, appearing the reducing power to Benedict's and red tetrazolium solution. The periodate oxidation of this demethyl compound yielded no formaldehyde.

The furfural like substance, resulted from compound B by refluxing it with 4N hydrochloric acid, showed a definite brown spot at R_F 0.45 by spraying with ninhydrin solution on the paper chromatogram (solvent: *n*-butanol, acetic acid, water, 4:1:2.5 V.). This substance was eluted from the strips with water, and determined by the ultraviolet absorption.

The hydrochloride of this new compound (compound D) was obtained as crystalline form after purification of the hydrolyzates of compound B with cellulose powder chromatography. Its ultraviolet absorption spectra in 0.1N HCl and 0.1 N NaOH solutions are shown in Fig. 7[10]. These spectra indicated that the basic function had a

10) The bathochromic shift from acidic to basic medium is interpretable on the basis of π-σ-*p*-conjugation※ in the following way. In the basic medium, aminomethylfurfural (I) has the unshared *p*-electrons of nitrogen atom and is closely analogous to hydroxymethyl furfural (II), which also has a lone pair of oxygen atom. In the acidic medium, a lone pair of amino nitrogen atom is coordinated with proton and forms an ammonium cation (III), so its spectrum must be more closely related to 5-alkylfurfural or furfural (IV). The absorption of these compounds were shown in next table.



I. II. III. IV.

Compound	λ max	ε×10⁻⁴	λ max	ε×10⁻⁴
I	289.5 mμ	1.62	228	0.37
II	285	1.65	228	0.36
III	278	1.54	228	0.35
IV	278	1.50	225	—

※) E. A. Braude, Determination of organic structure by physical methods, p. 156 (1955), Academic Press.

pKa′ 8.1[11].

The infrared absorption (Fig. 8) was characterized by intense bands at 1534, 1033, 962,

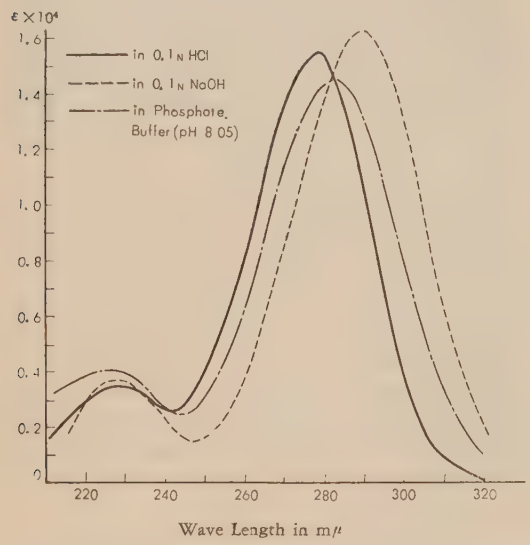


FIG. 7. Ultraviolet Absorption Spectra of Aminomethylfurfural

to -OH group was not observed. The presence of aldehyde group was observed by the infrared absorption (at 1684 cm^{-1}) and the formation of N-acetyl-2,4-dinitrophenylhydrazone.

It was found that treatment of the compound D with nitrous acid produced a compound (IV), which had identical R_F values on paper chromatograms, the same ultraviolet absorption spectrum and the same color reactions with that of 5-hydroxymethylfurfural[13] prepared from sucrose. 2,4-Dinitrophenylhydrazone of this deaminated product had also the same mixed m.p. and the same infrared absorption spectrum to that of 5-hydroxymethylfurfural 2,4-dinitrophenylhydrazone.

On the basis of these data, compound D is said to be 5-aminomethylfurfural (III) and compound B to be one of the stereoisomeric forms of methyl-6-amino-6-deoxy-aldohexopyranoside.

Compound B gave D-aldohexose by nitrous acid deamination and following hydrolysis which was isolated and identified as D-glucose-phenylosazone. Therefore compound B would

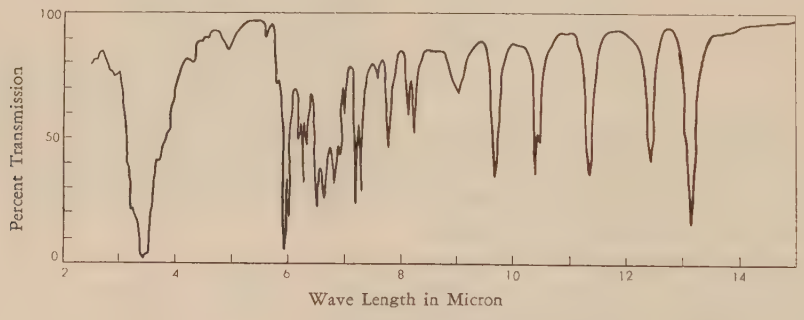


FIG. 8. Infrared Spectrum of Aminomethylfurfural in Nujol

955, 806 and 760 cm^{-1}. These bands were due to the presence of substituted furan ring[12] (5-hydroxymethylfurfural also gave the absorptions at 1534, 1021, 968, 950, 810 and 769cm^{-1}). The bands at 1608 and 1580 cm^{-1} indicated the presence of $-NH_3^+$ group, but the band due

be either methyl-6-amino-6-deoxy-D-glucopyranoside or methyl-6-amino-6-deoxy-D-mannopyranoside.

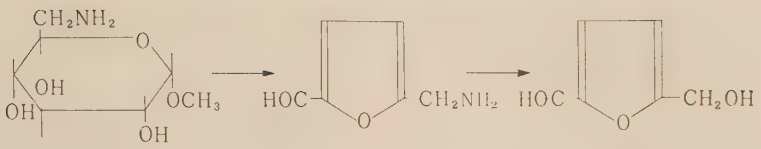
The stereochemical structure of compound B would be also suggested from its infrared absorption and molecular rotation as follows.

In the infrared spectrum of compound B, the following strong absorptions were observed

11) a. L. A. Flexser, L. P. Hammett and A. Dingwall, *J. Am. Chem. Soc.*, **57**, 2103 (1935).
b. L. A. Flexser and L. P. Hammett, *ibid.*, **60**, 885 (1938).

12) a. M. Yamaguchi, *Japan Analyst*, **7**, 210 (1958).
b. A.H.J. Cross, S.G.E. Stevens and T.H.E. Watts, *J. Appl. Chem.*, **7**, 562 (1957).

13) W. N. Haworth and W.G.M. Jones, *J. Chem. Soc.*, **1944**, 667.



II III IV

in the region of 1000~700 cm^{-1}; 977, 946, 899, 813 and 754 cm^{-1}. The absorptions at 899, 813 and 754 cm^{-1} may be type 1, 2a and 3[14], and the corresponding absorption at 870, 832 and 751 cm^{-1} were observed for N-acethyl compound B. From the data of S.A. Baker and coworkers[14], these absorptions may suggest that compound B is the derivative of α-D-glucopyranose.

by the synthesis. Methyl-6-amino-6-deoxy-α-D-glucopyranoside was synthesized starting from methyl-α-D-glucopyranoside and by treating methyl-6-O-tosyl-α-D-glucopyranoside with ammonia[16]. These two compounds were found to be identical by infrared spectra, optical rotations, melting points and paper chromatograms.

So, compound B should have structure II.

TABLE I

Substance	$[\alpha]_D$	Molecular rotation in water
Compound B hydrochloride	+133°	$+302\times10^2$
Compound B N-acetate	+135°	$+317\times10^2$
Methyl-α-D-glucopyranoside	+158.9°	$+308\times10^2$
Methyl-6-O-methyl-α-D-glucopyranoside	+127.9°	$+268\times10^2$ [15]
Methyl-6-chloro-6-deoxy-α-D- //	+139.7°	$+297\times10^2$ [15]
Methyl-6-bromo-6-deoxy-α-D- //	+107.4°	$+276\times10^2$ [15]
Methyl-6-iodo-6-deoxy-α-D- //	+ 93.9°	$+285\times10^2$ [15]
Methyl-6-iodo-6-deoxy-α-D- //	+101.5°	$+308\times10^2$ [15]
Methyl-α-D-glucopyranoside-6-O-tosylate (2.5 H_2O)	+ 69.7°	$+274\times10^2$ [15]
Methyl-α-D-glucopyranoside-6-O-sulfate (Ba salt)	+ 81°	$+278\times10^2$ [15]

Comparison of the molecular rotations of methyl aldohexopyranoside with those of the corresponding C_6-substituted derivatives, revealed that replacement of a hydroxyl at C_6 by other groups did not result any significant numerical change. As shown in Table I, the molecular rotations of compound B hydrochloride and its N-acetate were very similar to those of methyl-α-D-glucopyranoside and its C_6-substituted derivatives.

From the above results, compound B was suggested to be methyl-6-amino-6-deoxy-α-D-glucopyranoside. This conclusion was confirmed

14) S. A. Baker, E. J. Bourne and D. H. Whiffen, Methods of Biochemical Analysis, Vol. 3, p. 213 (1954), Interscience Publishers.

Compound C (Methyl-3-amino-3-deoxy-α-D-glucopyranoside)

Compound C was the third product isolated by methanolysis of kanamycin, and was obtained as a crystalline free base. Analytical data was in agreement with the molecular formula $C_7H_{15}NO_5$. The presence of one methoxyl group was indicated by a Zeisel determination. The basic group was shown

15) G. N. Bollenback, Methyl Glucoside, p. 74~145 (1958), Academic Press.

16) In this synthesis it is considered that even if 3:6 anhydro ring is formed from 6-tosylglucopyranoside this anhydro ring is stable to ammonia, (5:6 epoxy ring formation is impossible), so the possibility of formation of amino sugars from this anhydro ring should be denied.

by potentiometric titration to be an order of basic strength almost equivalent to glucosamine. It had positive ninhydrin but negative reducing sugar test.

The infrared absorption spectrum (Fig. 9) suggested that this compound was also methyl-amino-deoxy-aldohexopyranoside.

chain structure, and indicated that compound C was methyl-3-amino-3-deoxy-α-D-aldohexopyranoside.

Further the hydrochloride of demethyl C phenylosazone, $C_{18}H_{23}N_5O_3 \cdot HCl$, was obtained in crystalline form, and since this phenylosazone (VII) had primary amino group, it was clear

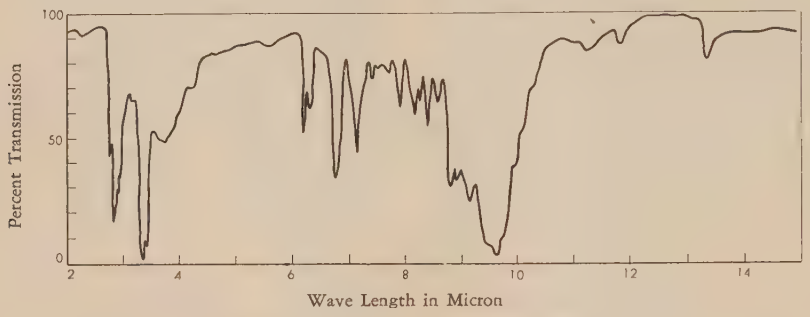


FIG. 9. Infrared Spectrum of Compound C Base in Nujol.

On hydrolysis of compound C with 2N hydrochloric acid for 7 hours, C was converted into its demethyl compound which showed R_F 0.32 on paper chromatogram (solvent; *n*-butanol, acetic acid, water), and had positive reducing sugar tests and Elson-Morgan reaction.

Treatment of compound C with acetic anhydride and Dowex-1 (CO_2 form) in water gave needle crystals of N-acetyl derivative.

By the periodate oxidation, compound C consumed 2 moles of periodate without formation of formaldehyde, while N-acetyl derivative was not attacked by periodate.

Treatment of compound C with sodium periodate (2 moles) yielded a dialdehyde. As this dialdehyde was syrup and inadequate for identification, it was further oxidized to the diacid. The strontium salt of this acid (VI) was crystalline and showed identical infrared spectrum and optical rotatory power with strontium D'-methoxy-D-(hydroxymethyl)-diglycolate[17] prepared from methyl-α-D-glucopyranoside. The above facts excluded any branched

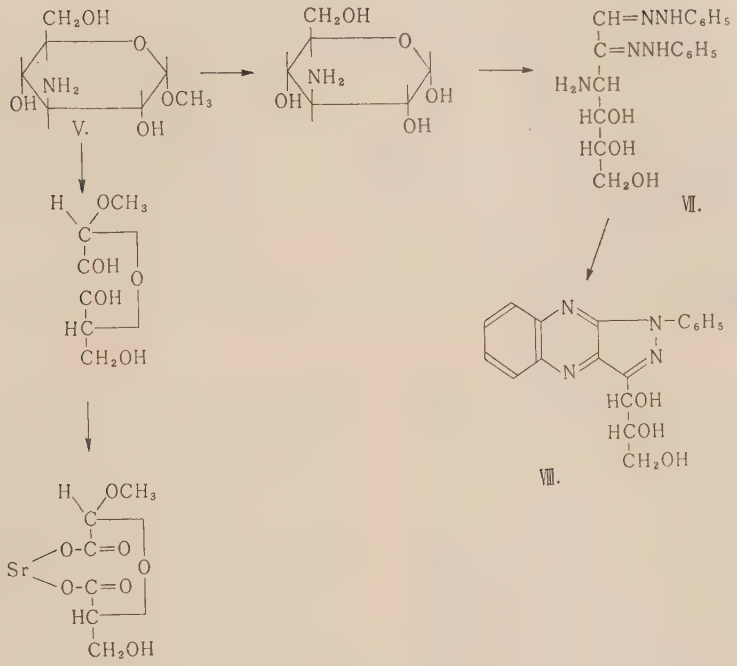
17) E. L. Jackson and C. S. Hudson, *J. Am. Chem. Soc.*, **59**, 994 (1937).

that compound C was not methyl-2-amino-2-deoxy-aldohexopyranoside.

Deamination of demethyl C with phenylhydrazine in the presence of O-phenylenediamine and acetic acid gave 1-phenylflavazole derivative (VIII), which was identified as D-erythro-trihydroxypropyl-1-phenylflavazol by comparing with authentic sample[18]. The acetyl derivative of VIII gave identical infrared spectrum and melting point with that of triacetyl derivative of D-glucose phenylflavazol. As this phenylflavazol derivative had no primary amino group, the location of amino group in compound C was definitely determined at C_3, and these results indicated that the configuration of C_4 and C_5 were all D. Thus compound C should have D-glucose, D-altrose, D-mannose or D-allose configuration.

The high levorotatory character of demethyl C phenylosazone hydrochloride in the mixture of pyridine and 50% methanol suggested that this compound might be D-glucose or D-mannose derivative. For, in the same solvent, phenylosazone of D-galactose was dextrorotatory but

18) H. Ohle and R. Liebig, *Ber.*, **75**, 1537 (1942).



VI.

phenylosazone of L-sorbose, D-glucose and D-psicose were all levorotatory. And the rotatory power of demethyl C phenylosazone was more approximate to that of D-glucose phenylosazone than to that of L-sorbose phenylosazone or D-psicose phenylosazone. (c.f. the previous paper[6d])

The molecular rotations and infrared absorption spectra of compound C and its derivatives also suggested that compound C was a glucose derivative, as follows.

The molecular rotations of compound C ($[M]_D + 263 \times 10^2$) and N-acetyl compound C ($[M]_D + 372 \times 10^2$) approximated those of glucose derivatives but differed from those of mannose and allose. (c.f. the previous paper[6d])

In the infrared spectrum of compound C, the following strong absorptions were observed in the region of 1000~700 cm^{-1}; 890, 848 and 750 cm^{-1}, which suggested the derivative of α-D-glucopyranose[14]. The absence of absorption type 2c at ca. 875 cm^{-1} indicated that compound C was not the derivative of D-galactopyranose and D-mannopyranose[14]. The infrared absorptions of compound C, its derivatives and related compounds are show in Table II.

To confirm these data, methyl-3-amino-3-deoxy-α-D-glucopyranoside tetraacetate was synthesized from 3-*p*-toluenesulfonyl-α-D-methylglucoside triacetate according to Peat and Wiggins[19]. This synthetic product and compound C tetraacetate were identical in their rotatory powers, m.p., mixed m.p. and infrared absorption spectra. Thus, compound C was established as methyl-3-amino-3-deoxy-α-D-glucopyranoside (V).

THE STRUCTURE OF KANAMYCIN

The above results indicated that the molecule of kanamycin, $C_{18}H_{36}N_4O_{11}$, was composed of deoxystreptamine, 6-amino-6-deoxy-D-glucose and 3-amino-3-deoxy-D-glucose, which were glycosidically linked together. In order to determine the position of the linkage between

19) S. Peat and L. F. Wiggins, *J. Chem. Soc.*, **1938**, 1810.

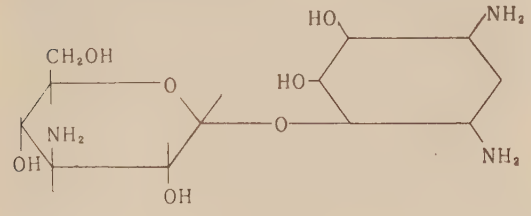
TABLE II. INFRARED SPECTRA OF COMPOUND C, ITS DERIVATIVES AND RELATED COMPOUNDS IN NUJOL

	Frequencies (cm^{-1})		
	Type 1	Type 2a	Type 3
Compound C	890 (m)	848 (m)	750 (m)
Methyl-α-D-glucopyranoside	896 (s)	840 (s)	745 (s)
Compound C N-acetate	910 (s)	849 (m)	745 (s)
Methyl-2-amino-2-deoxy-α-D-glucopyranoside	903 (s)	848 (m)	762 (s)
Compound C tetraacetate	895 (s)	858 (s)	763 (m)
Methyl-α-D-glucopyranoside tetraacetate	896 (m), 887 (m)	855 (m)	758 (m)

s: strong, m: medium

the above moieties, partial hydrolysis, methylation, and periodate oxidation of kanamycin were carried out.

Kanamycin was hydrolyzed partially in 6N hydrochloric acid at 37° for 11 days, and the separation of hydrolyzates by cellulose powder chromatography gave a crystalline hydrochloride, having the formula $C_{12}H_{25}N_3O_7 \cdot 3HCl$ (compound F). The free base and N-acetyl derivative were prepared. Methanolysis and hydrolysis products showed it to be O-3-amino-3-deoxy-D-glucopyranosyl-2-deoxystreptamine (IX). The location of glucosidic linkage between 2-deoxystreptamine and 3-amino-3-deoxyglucose was examined by periodate oxidation, and it was found that the hydrochloride and the free base consumed ca. 4 moles of periodate, while N-acetyl derivative consumed 1 mole. These facts indicated that 3-amino-3-deoxy-D-glucopyranose linked to 1,3-diamino-4,5,6-trihydroxy cyclohexane at C_4 or C_6 position.



IX.

This compound was almost inactive against *B. subtilis* (lower than one-hundredths of kanamycin).

During the hydrolysis of kanamycin in 6N hydrochloric acid at 37°, it was observed that 6-amino-6-deoxy-D-glucose was liberated faster than 3-amino-3-deoxy-D-glucose (determined by paper chromatography). This phenomenon coincided to the fact that methyl-6-amino-6-deoxy-α-D-glucopyranoside could easily be converted to its demethyl compound in the weaker acid and at the lower temperature than methyl-3-amino-3-deoxy-α-D-glucopyranoside. This fact is well understood from the different shielding effect of $-NH_3^+$ group, attached in the different position in the amino sugars, on the glycosidic linkage against hydrolysis.

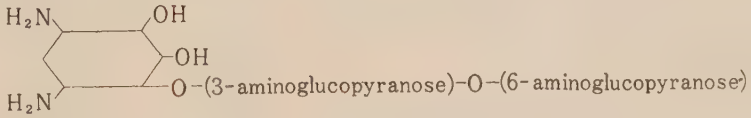
The reaction of kanamycin with sodium nitrite in the presence of acetic acid was also examined and deamination and simultaneous hydrolysis of glycosidic bonds were observed[20]. Paper chromatography (solvent: *n*-butanol, acetic acid, H_2O) of the deaminated products, visualized by aniline hydrogen phthalate, revealed two spots, one of which was hexose sugar having R_F 0.27, the other was probably anhydrosugar having R_F 0.45. The hexose fraction was isolated as phenylosazone and identified as D-glucose phenylosazone.

The remaining problem was to determine the site of the linkage, joining 6-amino-6-deoxyglucose to the other moiety in kanamycin. The preliminary evidence was obtained by periodate oxidation of kanamycin and N-acetyl

20) Analogous examples of cleavage of glycosidic linkage by nitrous acid were reported in methyl-2-glucosaminide, chitosan and ϕ-heparin (A. B. Foster, E. F. Martlew and M. Stacey, *Chem. & Ind.*, **1953**, 825).

kanamycin.

N-acetylkanamycin consumed 2 moles of periodate with formation of 1 mole of formic acid and then the oxidation ceased. After the periodate oxidation of N-acetylkanamycin, the oxidation product was degraded with methanolic hydrogen chloride. The paper chromatogram of this degradation products showed the presence of deoxystreptamine and compound C, and deoxystreptamine was isolated. The paper chromatography and ultraviolet absorption indicated that the compound B was destroyed by the periodate.



X.

These facts excluded the structure X for kanamycin, because N-acetylderivative of X should consume 3 moles of periodate and deoxystreptamine moiety should be destroyed.

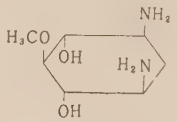
And these results also indicated that aminohexoses in kanamycin molecule had pyranose form.

Kanamycin free base in the solution of 0.02M periodic acid (pH 1.8) consumed 4.3 moles of periodate within about 5 hours and the reaction essentially completed. But in the acetate buffer of pH 4.7, kanamycin free base consumed 4.4 moles within 5 hours and then gradually increased the consumption of periodate to 5.2 moles in 24 hours, 6.3 moles in 48 hours and 7.4 moles in 95 hours.

The linkage between 6-amino-6-deoxy-D-glucose and 2-deoxystreptamine was determined by the methylation of kanamycin.

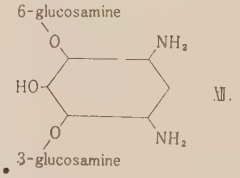
N-Acetylkanamycin was methylated with dimethyl sulfate and sodium hydroxide[5]. A single methylation gave a product, in which, judging from its methoxyl content, the six hydroxyl groups were replaced by methoxyl. But the basic character and positive ninhydrin reaction of the methylated product suggested that the acetylated amino groups had been partially degraded to amino groups in the strongly alkaline methylation medium. After the re-N-acetylation, this compound was methylated further twice with silver oxide and methyl iodide. The methylated product was hydrolysed with boiling 2N hydrochloric acid for 5.5 hours and the solution was concentrated to a small volume in vacuo and ethanol was added, yielding crystalline product. From analytical data and infrared spectrum, this compound was shown to be diaminodihydroxymonomethoxycyclohexane dihydrochloride. Since this compound had no optical activity, the methoxyl group should be in the plane of symmetry of the molecule, and the methoxyl group should be at C_5 of 2-deoxystreptamine (1,3-diamino-4,5,6-trihydroxycyclohexane.).

This fact was also supported by the periodate oxidation of N,N′-diacetyl derivative of this compound, which consumed only 0.3 mole of periodic acid for 48 hours. So this substance have the structure XI.



XI.

Thus, the structure of kanamycin was postulated as XII.



XII.

In consideration with the configuration of

TABLE III. SPECIFIC AND MOLECULAR ROTATIONS OF KANAMYCIN, ITS DEGRADATION COMPOUNDS, AND MODEL COMPOUNDS

	$[\alpha]_D$※)	$[M]_D$
Compound A dihydrochloride	0	0
Compound B hydrochloride	+133°	$+305\times10^2$
Methyl-6-amino-6-deoxy-β-D-glucopyranoside-hydrochloride	−25.1°[25]	-57×10^2
Compound C free base	+136°	$+263\times10^2$
Methyl-3-amino-3-deoxy-β-D-glucopyranoside	−47.4°[19]	-91×10^2
Compound F trihydrochloride	+69.9°	$+303\times10^2$
Kanamycin free base	+146°	$+708\times10^2$
Kanamycin monosulfate	+120°	$+700\times10^2$
D-1-O-α-D-Galactopyranosyl *myo*-inositol	+135.6°[26]	$+512\times10^2$
D-1 (or L-1)-Methyl *myo*-inositol (*d*-bornesitol)	+30.6°[27]	$+59\times10^2$
D-4 (or L-4)-Methyl *myo*-inositol (*d*-ononitol)	+6.6°[28]	$+13\times10^2$
Methyl-α-D-galactopyranoside	+196°	$+380\times10^2$
α,α-Trehalose dihydrate	+178.3°[29]	$+675\times10^2$
α-D-Glucopyranoside-2-amino-2-deoxy-α-D-glucopyranoside hydrochloride	+176°[30]	$+665\times10^2$
β,β-Trehalose (Isotrehalose)	−41.5°[29]	-141×10^2
α,β-Trehalose (Neotrehalose)	+95°[29]	$+325\times10^2$

※) All values were for aqueous solutions.

glucosidic carbon in O-3-amino-3-deoxy-D-glucopyranosyl-2-deoxystreptamine (compound F), the following literature came into reference. W. Klyne[21] reported that in the steroid 3-glycopyranosides, the rotation contribution of the carbohydrate component was almost independent of the nature of the steroid component and was very approximately equal to $[M]_D$ of the corresponding α-or β-methylglycopyranoside. This principle was applied by Reichstein and his colleagues to cardiac glycosides. Besides, the configurations of oligosaccharides have been determined by the application of the Hudson's isorotation rules, as shown for the α-configuration of the glucopyranose moiety in sucrose[22].

Appling this general rule to cyclitol glycopyranoside[23] and compound F, the rotatory contribution of optically active deoxystreptamine portion may be $+394\times10^2$ or $+40\times10^2$, corresponding to β or α glycosidic linkage (Table III). Since the molecular rotation of *d*-ononitol (4-methyl *myo*-inositol)[24] is small ($[M]_D$ $+13\times10^2$), the large molecular rotation ($+394\times10^2$) for deoxystreptamine portion may be unprobable and α-linkage was suggested.

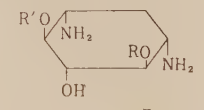
The α-configuration was also confirmed by the three absorptions, characteristic of α-glucopyranose, in the 950~730 cm^{-1} region of the infrared spectra of compound F trihydrochloride, its N-triacetate, and also compound C (Table IV). Since compound F was isolated by mild hydrolysis, it is natural to consider that

21) W. Klyne, *Biochem. J.*, 47 xli (1950).

22) M. L. Wolfrom and F. Shafizadeh, *J. Org. Chem.*, **21**, 88 (1956).

23) In the case of D-1-galactopyranosyl *myo*-inositol (I), the rotatory contribution of the optically active *myo*-inositol portion is about $[M]_D+130\times10^2$ [$[M]_D$ of (I) $(+510\times10^2)-[M]_D$ of methyl-α-D-galactoside $(+380\times10^2)$] which is the same order of magnitude as $[M]_D(+59\times10^2)$ of *d*-bornesitol (1-methyl-*myo*-inositol), and this difference is much smaller than the difference of $[M]_D(380\times10^2)$ between anomeric methyl α- or β-D-galactopyranoside.

24) The optical contribution of deoxystreptamine portion may be regarded as not so much different from 4-methyl-2-deoxystreptamine (II) which may be approximately the same order as that of 4-methyl *myo*-inositol (III) (i.e. *d*- or *l*-ononitol).



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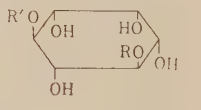


III.

for D-4-substituted compounds	for L-4-substituted compounds
II R=H, R'=CH_3	R=CH_3, R'=H
III R=H, R'=CH_3	R=CH_3, R'=H

25) E. Fischer and K. Zach, *Ber.*, **44**, 132 (1911).

26) R. J. Brown and R. F. Serr, *J. Am. Chem. Soc.*, **75**, 1040 (1953).

27) a. A. B. Foster and M. Stacey, *Chem. & Ind.*, **1953**, 279. *C. A.*, **48**, 3275 (1954).
b. S. J. Angyal, P. T. Gilham and C. G. Macdonald, *J. Chem. Soc.*, **1956**, 1417.

28) V. Plouvier, *Compt. Rend.*, **241**, 983 (1955): *C. A.*, **50**, 11959 (1956).

29) K. Aso, *J. Agr. Chem. Soc. Japan*, **31**, A56 (1957).

30) F. Arcamone and F. Bizioli, *Gazz. Chem. Ital.*, **87**, 896 (1957).

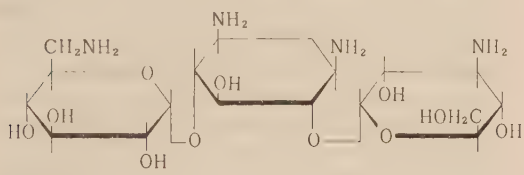
the α-D-glucosidic linkage in compound F could not be altered by these hydrolysis Therefore the remaining linkage between 2-deoxystreptamine and 6-glucosamine should be also α-D-configration (Table III)[31] from the comparison of molecular rotation of kanamycin with that of compound F.

Infrared spectra of kanamycin, its acetyl derivatives, its degradation products and related compounds are shown in Table IV. Kanamycin and its derivatives show absorption at 810~850 cm^{-1}, which are characteristic of α-glucopyranosidic linkage. Comparison of these spectra shows that the absorption at ca. 815 cm^{-1} of kanamycin free base and its monosulfate is indicative of α-D-6-aminoglucopyranosidic linkage, whereas the absorption at ca. 839 cm^{-1} is due to α-D-3-aminoglucopyranosidic linkage.

From all these data, the structure XV or XVI are assigned to kanamycin.

EXPERIMENTAL

Melting points reported herein were uncorrected. Infrared spectra were measured by the Nujol mull method (solid) and liquid film method (liquid) with



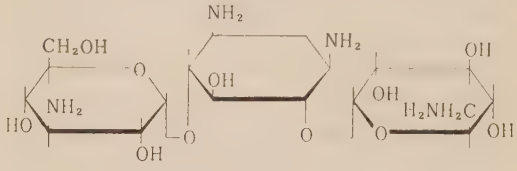


Table IV. Infrared Absorptions of Kanamycin and Related Compounds in Nujol

	Frequencies (cm^{-1})※						
	other peaks	Type 1[14]	Ring methylene[14]	Type 2a[14]		other peaks	Type 3[14]
A dihydrochloride			863 (s)				721 (w)
B hydrochloride		899 (m)			813 (m)		754 (s)
B N-acetate	909 (m)	870 (s)			832 (m)		751 (s)
B tetraacetate		896 (s)			861 (s)		750 (m)
C free base		890 (m)		848 (m)			750 (m)
C N-acetate		910 (s)		849 (m)			745 (s)
C tetraacetate		895 (s)		858 (s)			763 (m)
F trihydrochloride	917 (mb)	888 (m)	872 (s)	860 (m)			769 (wb)
F N-triacetate		920 (m)	862 (m)	847 (m)		757 (mb)	730(m), 714(mb)
Kanamycin free base	917 (s)	891 (s)	848 (m)	836 (m)	817 (m)	775 (s)	758 (s)
// monosulfate	939 (s)	879 (m)	871 (m)	842 (m)	812 (m)	778 (m)	757 (s)
// N-tetraacetate		910 (s)	868 (w)	844 (m)		780 (s)	735 (sb)
// polyacetate	931 (s)	904 (sb)	844 (wb)				763 (s)

※ w; weak, m; medium, s; strong, b; broad

31) Deducing from what cited in 24), the rotatory contribution of $C_{4,6}$-disubstituted 2-deoxystreptamine which is disubstituted symmetrically to the plane of symmetry, for the molecular rotation of kanamycin is considered to be smaller than that of glucosamine portions, therefore $[M]_D$ of kanamycin may be able to compare with those of 1,1-trehalose and its derivatives (Table III). As $[M]_D$ value of kanamycin ($+708\times10^2$) is approximately equal to that of α,α-trehalose ($+675\times10^2$) and its amino derivatives ($+665\times10^2$), α,α-configuration in kanamycin should be suggested.

Kōken DS 301 spectrometer. Ultraviolet measurements were made with a Hitachi EPU-2 spectrometer.

Kanamycin monosulfate — Kanamycin monosulfate was prepared according to K. Maeda et al.[2b], and recrystallized from water-methanol (dried at 150°C in vacuo). m.p. 260~270° (dec.), $[\alpha]_D^{29}$ +120° (c, 0.456 in water)

Anal. Calcd. for $C_{18}H_{36}N_4O_{11}\cdot H_2SO_4$: C, 37.1; H,

6.6; N, 9.6; S, 5.5.

Found: C, 36.7; H, 6.2; N, 9.2; S, 5.4; OCH_3, 0.58

Kanamycin free base—Kanamycin free base was prepared from the monosulfate by treating with Amberlite IRA 400 (OH), and recrystallized from water-methanol-ethanol. m.p. ca. 263~268° (dec.), $[\alpha]_D^{29}$ + 146° (c, 0.669 in water).

Anal. calcd. for $C_{18}H_{36}N_4O_{11}$: C, 44.6; H, 7.5; N, 11.6; neut. eq., 121.1.

Found: C, 44.1; H, 7.8; N, 10.8 (Dumas), 10.8 (Van Slyke); neut. eq., 120.

Tetra N-acetylkanamycin—To a solution of 800 mg of kanamycin free base in 100 ml of methanol, was added 3.5 ml of acetic anhydride and allowed to stand at room temperature for 3 hr. The reaction mixture was concentrated in vacuo and allowed to crystallize. Twice recrystallization from water-methanol yieded 450 mg. m.p. 223° (dec.). $[\alpha]_D^{29}$ +110° (c, 0.629 in water).

Anal. Calcd. for $C_{18}H_{32}N_4O_{11}\cdot 4(CH_3CO)$: C, 47.9; H, 6.8; N, 8.4; acetyl (monohydrate), 25.7.

Found: C, 47.0; H, 7.0; N, 8.3; acetyl (monohydrate), 24.4; C-CH_3 (monohydrate), 24.9.

Paper chromatography of this compound showed a single spot at R_F 0.30 (solvent system: *n*-butanol, pyridine, water 6:4:3, sprayed with sodium hypochlorite-iodide-starch[32]).

Tetra N-benzoylkanamycin—Kanamycin monosulfate (2 g) was dissolved in 30 ml of water and treated with 8.5 g of benzoylchloride and 40 ml of 20% sodium hydroxide solution. After stirring for 1 hr at room temperature, the resulting precipitate (2.8 g) was collected by filtration. This compound was dissolved in 300 ml of methanol and refluxed for 4 hr, after the addition of 10% methanolic sodium hydroxide (13 ml). The solution was adjusted to pH 7.0 with hydrochloric acid and concentrated in vacuo to crystallize. Yield, 2.0 g. A sample for analysis was recrystallized from the mixture of dimethylformamide, methanol and water. m.p. 278~282° (dec.).

Anal. Calcd. for $C_{46}H_{52}N_4O_{15}$: C, 61.3; H, 5.8; N, 6.2.

Found: C, 60.9; H, 6.1; N, 6.2.

Tetra N-*p*-dimethylaminobenzylidene kanamycin monosulfate—Kanamycin monosulfate (200 mg) in 5 ml of water was added with stirring to 5 ml of methanol solution containing 213 mg of *p*-dimethylaminobenzaldehyde. The solution was warmed to 50° for 10 minutes and allowed to stand at room temperature overnight. The white needle crystals formed were filtered and washed several times with methanol and water. Yield, 156 mg. m.p. 249~250° (dec.). Its infrared spectrum showed the absorption band of substituted phenyl ring, but no carbonyl absorption band of *p*-dimethylaminobenzaldehyde.

Anal. Calcd. for $C_{54}H_{72}N_8O_{11}\cdot H_2SO_4$: C, 59.2; H, 6.8; N, 10.2.

Found: C, 59.8; H, 6.6; N, 10.4.

Countercurrent distribution of kanamycin—Kanamycin tetrahydrochloride was distributed between the next solvent system.

The upper layer: The mixture of 475 ml of *n*-butanol and 25 ml of ethyl acetate. The lower layer: 10 g of sodium lauryl sulfate and 5 g of sodium chloride in 500 ml of water.

The distribution was carried out in a 31-plate Craig apparatus. The above solvents were equilibrated and introduced into the apparatus. Fifty mg of kanamycin tetrahydrochloride was distributed.

After 31 transfers, the antibiotic contents of each phase in each tube was determined. When total concentration per tube was plotted against the corresponding tube number, a typical distribution curve was obtained (Fig. 10). This curve showed no detectable deviation from a calculated curve.

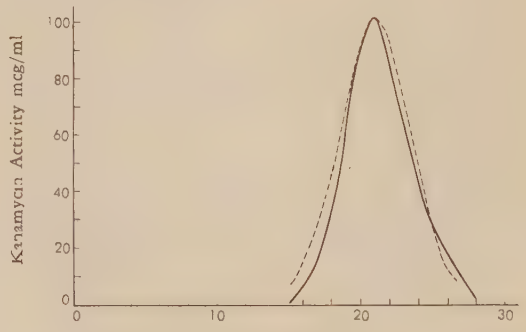


FIG. 10. Countercurrent Distribution of Kanamycin.
—— Experimental ---- Theoretical

2-Deoxystreptamine (Compound A)—Kanamycin tetrahydrochloride (4.5 g) was refluxed in 3 *l* of methanol saturated with hydrogen chloride (twice saturated with hydrogen chloride gas during the methanolysis) for 8 hours. The solution was concentrated in vacuo to one-fourth volume, separating precipitate. Yield, 1.57 g. This precipitate was crude deoxystreptamine hydrochloride.

Deoxystreptamine dipicrate—A solution of 60 mg

32) S. C. Pan and J. D. Dutcher, *Anal. Chem.*, **28**, 836 (1956).

crude deoxystreptamine hydrochloride in 0.5 ml of water was mixed with a solution of picric acid (100 mg) in 2 ml of hot water. After cooling, needle crystals of deoxystreptamine dipicrate separated; yield, 100 mg. The product was recrystallized from water; yield, 87 mg. It decomposed partially at ca. 210° and completely between 255~260°.

Anal. Calcd. for $C_6H_{14}N_2O_3(C_6H_3N_3O_7)_2$: C, 34.9; H, 3.2; N, 18.1.

Found: C, 35.3; H, 3.3; N, 17.2.

The content of picric acid was determined by the ultraviolet absorption at 360 mμ (in 95% ethanol). Found: 75%, Calcd.: 73.9%.

Deoxystreptamine dihydrochloride — Deoxystreptamine dipicrate (130 mg) was suspended in 2 ml of methanol, 0.12 ml of conc. hydrochloric acid was added under stirring, and converted to the hydrochloride. The crystals of the hydrochloride were recovered by filtration, and washed with acetone and ethyl ether. The crude crystals were dissolved in water and ethanol was added, separating needle crystals. Yield 25 mg. The crystals decomposed partially between 213~215° and completely between 240~250°. $[\alpha]_D^{30}0$ (c, 0.8 in water)

Anal. Calcd. for $C_6H_{14}N_2O_3 \cdot 2HCl$: C, 30.7; H, 6.9; N, 11.9, eq. wt. 117.5.

Found: C, 30.7; H, 6.7; N, 11.8, eq. wt. 120 (potentiometric titration).

Deoxystreptamine monohydrochloride—To a solution of deoxystreptamine dihydrochloride (50 mg) in water, adjusted to pH 10 by adding triethylamine, ethanol was added, precipitating plate crystals. Yield, 36 mg. The crystals showed no definite melting point and decomposed between 250~270°.

Anal. Calcd. for $C_6H_{14}N_2O_3 \cdot HCl$: C, 36.3; H, 7.6; N, 14.1.

Found: C, 35.6; H, 7.31; N, 13.8.

Electrometric titration of this compound with 0.1 N hydrochloric acid showed that the molecular weight was ca. 200.

Calcd. for $C_6H_{14}N_2O_3 \cdot HCl$: mol. wt. 198.5.

Pentaacetyl deoxystreptamine A mixture of acetic anhydride (2 ml) pyridine (4 ml) and deoxystreptamine dihydrochloride (100 mg) was refluxed for thirty minutes, and then evaporated. The residue, dissolved in chloroform, was washed with water, dried, concentrated to a small volume and mixed with ether, causing the separation of plate crystals. Yield, 30 mg. Recrystallized from chloroform-ether. m.p. 190~192°.

Anal. Calcd. for $C_3H_9N_2O_3(CH_3CO)_5$: C, 51.6; H, 6.5; N, 7.5.

Found: C, 51.3; H, 5.7; N, 6.6.

N, N′-Dibenzoyl deoxystreptamine — A mixture of 60 mg of deoxystreptamine dihydrochloride, 0.5 ml of pyridine and 0.4 ml of benzoyl chloride was heated just to boiling, then cooled. The crystals were recovered by filtration and washed with ethyl ether, and then were refluxed with 0.5 N sodium hydroxide in methanol for 5 hr. The needle crystals were obtained. Yield, 50 mg. Recrystallized from dimethylformamide methanol. m.p. 282~283°.

Anal. Calcd. for $C_{20}H_{22}N_2O_5$: N, 7.6.

Found: N, 7.1.

Periodate oxidation of deoxystreptamine—To an aqueous solution containing 23.5 mg (0.1 millimole) of deoxystreptamine dihydrochloride and 0.2 g of sodium bicarbonate, 2.0 ml of 0.278 M aqueous periodic acid was added, and was immediately diluted to a total volume of 25 ml with water. Aliquots of 5 ml each were withdrawn at intervals for titration. Two ml of 0.1 M sodium arsenite solution and 1 ml of 20% potassium iodide were added to each aliquot before titration, and, after 10~15 minutes, the solution was titrated with standard 0.1 N iodine solution. Four moles of periodic acid per mole of deoxystreptamine were consumed within about thirty minutes, and the reaction was essentially complete.

N, N′-dibenzoyl deoxystreptamine consumed 2 moles of periodate per mole after about thirty hours, at the end of the reaction.

Methyl-6-amino-6-deoxy-α-D-glucopyranoside hydrochloride (Compound B hydrochloride) — Kanamycin tetrahydrochloride (4.5 g.) was degraded with HCl-methanol.

The solution was concentrated and the precipitate containing deoxystreptamine, was removed as described under "2-Deoxystreptamine (Compound A)". The mother liquor was reduced to a syrup in vacuo and was dissolved in 10 ml of methanol. White crystalline product (1.05 g) was obtained by adding 8 ml of ether. Recrystallized from methanol. m.p. 188° (dec.).

$[\alpha]_D^{30}+133°$ (c, 0.4755 in water)

Anal. Calcd. for $C_6H_{12}NO_4(OCH_3)HCl$: C, 36.6; H, 7.0; N, 6.1; OCH_3, 13.5; eq. wt. 229.

Found: C, 36.3; H, 7.1; N, 6.0; OCH_3 11.8; eq. wt. 220.

N-Acetate of compound B—Compound B hydrochloride (500 mg) and silver acetate (0.6 g) suspended in dry methanol containing 0.8 ml of acetic anhydride, and shaken for 4 hr. at room temperature. The solution was then heated for ten minutes at 60°, filtered,

concentrated to a syrup and crystallized from methanol-ethyl acetate. Yield, 350 mg. Recrystallized from methanol-ethyl acetate. m.p. 158~158.5°;

$[\alpha]_D^{19}$ +131° (c, 0.53 in methanol)

Anal. Calcd. for $C_7H_{14}O_5N(CH_3CO)$: C, 45.9; H, 7.3; N, 5.95; OCH_3; 13.2.

Found: C, 46.3; H, 6.75; N, 5.8; OCH_3, 13.1.

Periodate oxidation of compound B hydrochloride and its N-acetyl derivative — Twenty-four mg of sample was dissolved in water (10 ml) and sodium bicarbonate (40 mg) and 0.278 M aqueous periodic acid (2 ml) were added. The solution was immediately diluted to a total volume of 25 ml with 0.1 N acetate buffer (pH 4.7). Aliquots of 5 ml each were withdrawn at intervals for titration. Thirty mg of sodium bicarbonate, 2 ml of 0.1 M sodium arsenite solution and 1 ml of 20% potassium iodide were added and the solution was titrated with 0.1N standard iodine solution.

Consumed periodate per mole

	Time (hours)		
	3	5	22 hrs
B hydrochloride	1.9	1.9	2.2 mole
B N-acetate	1.8	1.7	1.9 mole

After periodate oxidation, formaldehyde content was determined with dimedone, but no formaldehyde was detected.

Demethyl B hydrochloride—One gram of compound B hydrochloride was dissolved in 40 ml of 1N-hydrochloric acid and refluxed for 6 hr. The solution was concentrated to syrup, was dissolved in a small amount of water and chromatographed on a cellulose column (120 ml of Whatman cellulose powder). After washing with a mixture of *n*-butanol, acetic acid, water (4:2:1) and elution with water, demethyl B hydrochloride was obtained as white powder. Yield, 200 mg. This compound showed a single spot (R_F 0.15) which gave positive ninhydrin and aniline hydrogen phthalate reactions on paper chromatogram.

After oxidation of this compound with excess periodate, formaldehyde was determined with dimedone. Demethyl B (1 mole) yielded 0.03 mole of formaldehyde, whereas glucosamine (1 mole) yielded 0.9 mole of formaldehyde in the parallel experiments.

Aminomethylfurfural hydrochloride (Compound D hydrochloride) — One gram of compound B hydrochloride was dissolved in 30 ml of 4 N hydrochloric acid and the solution was heated under reflux for 2 hr. The hydrolyzate was concentrated in vacuo to syrup, dissolved in a small amount of water and chromatographed on a cellulose powder column (Whatman) with *n*-butanol, acetic acid, water (4 : 2 : 1). The fractions containing the compound D were collected and extracted 3 times with water. The aqueous phase was washed with ether and ethyl acetate succesively, and concentrated in vacuo to dryness. A crystalline residue (230 mg) of aminomethylfurfural hydrochloride was obtained, and was recrystallized from *n*-propyl alcohol. White needle. m.p. 117~118° (dec.). The ninhydrin test on paper gave reddish yellow color. This compound is readily characterized by the R_F value (0.56) on paper chromatogram (solvent system: *n*-butanol, acetic acid, water 4 : 1 : 2.5 V.) and by ultraviolet spectrum, λ_{max}. 278 mμ ($\log \varepsilon$: 4.19), 228 mμ ($\log \varepsilon$: 3.54) in 0.1 N HCl, λ_{max}. 289.5 mμ ($\log \varepsilon$: 4.21), 228 mμ ($\log \varepsilon$: 3.57) in 0.1 N NaOH.

Anal. Calcd. for $C_6H_7NO_2 \cdot HCl$: C, 44.6; H, 5.0; N, 8.7.

Found: C, 45.9; H, 5.5; N, 8.0.

This compound was not stable and became gradually resinous by standing at room temperature. Aminomethylfurfural hydrochloride was converted to its N-acetate with acetic anhydride and silver acetate in methanol and was obtained as 2,4-dinitrophenylhydrazone, which recrystallized from 95% ethanol

Anal. Calcd. for $C_{14}H_{13}C_6N_5$: C, 48.5; H, 3.8; N, 20.1.

Found: C, 48.2; H, 3.8; N, 19.6.

The ultraviolet absorption in 95% ethanol was characterized by major maxima at 392 mμ ($\log \varepsilon$: 4.42) and at 302 mμ ($\log \varepsilon$: 3.85)[33]. In 0.25 N alkaline ethanol, the maxima appeared at 477 mμ ($\log \varepsilon$: 4.50). The infrared spectrum showed amide carbonyl band at 1645 cm^{-1}, amide NH-deformation band at 1558 cm^{-1}, CH bending mode of the furan ring at 1023 cm^{-1}.[33]

Deamination of Aminomethylfurfural — Aminomethylfurfural hydrochloride (110 mg) was dissolved in a mixture of water (5 ml) and 1 N-hydrochloric acid (1 ml), cooled to 0° and 150 mg of sodium nitrite was added. The reaction mixture was held at 5° for 40 hr. and extracted with ethyl acetate. The extract was washed with water and concentrated to dryness. Pale yellow oil (49 mg) was obtained. This compound gave identical ultraviolet spectrum, paper chromatogram[34] and the color reaction[35] with 5-hydroxymethylfurfural prepared from sucrose.

33) This band seemed to be the characteristic band of 2,4-dinitrophenylhydrazone of furan derivative. L. A. Jones, J. C. Holmes and R. B. Seligman, *Anal. Chem.*, **28**, 191 (1956).

34) A. Gottschalk, *Biochem. J.*, **52**, 454 (1952).

35) F. D. Snell and C. T. Snell, Colorimetric Methods of Analysis III, p. 276 (1957).

The product (45 mg) thus obtained by the deamination, was dissolved in water (4.5 ml) and was added with stirring to 2 N hydrochloric acid (8 ml) containing 2, 4-dinitrophenylhydrazine (110 mg). After 2 hr., the precipitate (61.3 mg) was separated by filtration and recrystallized from 95% ethanol. Yield, 43 mg. m.p. 182~183°.

Anal. Calcd. for $C_{12}H_{10}N_4O_6$: C, 47.1; H, 3.7; N, 18.35.

Found: C, 46.9; H, 3.6; N, 18.2.

The ultraviolet absorption spectra; λ_{max} 395 mμ (log ε: 4.48), 302 mμ (log ε: 3.32) (in 95% ethanol), and λ_{max} 479 mμ (log ε: 4.10) (in 0.25 N alkaline ethanol). The infrared spectrum showed CH bending mode of the furan ring at 1018 cm^{-1}.

Deamination of compound B hydrochloride—Compound B hydrochloride (500 mg) was dissolved in a mixture of 10 ml of water and 0.1 ml of conc. sulfuric acid, was cooled to 5°, and 500 mg of sodium nitrite was added. The solution was kept at 5° for 2 hr. and then at room temperature overnight. This reaction mixture showed reduction power (tested by red tetrazolium) and a weak single spot at R_F 0.52 on the paper chromatogram (solvent: *n*-butanol, acetic acid, water; visualized by aniline hydrogen phthalate). On refluxing this solution for 2 hr. with conc. sulfuric acid (0.25 ml), a new strong spot appeared at R_F 0.28 beside the spot of R_F 0.52. Considering from the R_F values, the spot R_F 0.28 would be aldohexose and the other probably anhydrohexose. The solution was neutralized with barium hydroxide and heated for 40 minutes on boiling water with phenylhydrazine (1 g) and acetic acid (600 mg). The resulting yellow needles (150 mg) was collected by filtration and recrystallized 4 times from ethanol-water: m.p. 202~4° (dec.). $[\alpha]_D^{19}$ −91° (c, 0.317 in dimethylsulfoxide).

Anal. Calcd. for $C_{18}H_{22}N_4O_4$: C, 60.3; H, 6.2; N, 15.6.

Found: C, 60.2; H, 6.2; N, 15.2.

This compound was identified as D-glucose phenylosazone by infrared spectrum and optical rotation.

Synthesis of methyl-6-amino-6-deoxy-α-D-glucopyranoside—A solution of 4.9 g of methyl-α-D-glucopyranoside in 25 ml of dry pyridine was cooled and treated by the portionswise addition of 5.2 g of tosylchloride, and the temperature was held below 20° during this reaction, and kept overnight at 10°. The reaction product was isolated by the ordinary way[36].

36) A. L. Raymond and E. F. Schroeder, *J. Am. Chem. Soc.*, **70**, 2788 (1948).

The resulting α-D-6-O-toluene-*p*-sulfonylmethylglucopyranoside (10 g) in methanol (55 ml) was saturated with dry ammonia at 7° and heated for 8 hr. at 100° in an autoclave. (The same product was also obtained by standing for 2 days at room temperature, 30°.) The reaction mixture was concentrated to a syrup, was dissolved in ethanol, mixed with ether and precipitates were removed by filtration. By adding concd. hydrochloric acid (1 ml) to the filtrate, separated crystals were filtered and washed with methanol.

Methyl-6-amino-6-deoxy-α-D-glucoypranoside, 1.8 g was obtained, (31% yield), and recrystallized from methanol. m.p. 188~9° (dec.). $[\alpha]_D^{30}$ +138° (c, 0.551 in water).

Anal. Calcd. for $C_7H_{16}NO_5 \cdot HCl$: C, 36.6; H, 7.0; N, 6.1.

Found: C, 36.6; H, 7.0; N, 6.1.

Dehydration of this synthetic product with 4 N hydrochloric acid at 100° also gave aminomethylfurfural identical to that from compound B.

Methyl-3-amino-3-deoxy-α-D-Glucopyranoside (Compound C)—Fifty grams of crystalline kanamycin monosulfate was refluxed in 3 l of methanol saturated with hydrogen chloride for 47 hr. The solution was concentrated in vacuo to 500 ml, and crystalline deoxystreptamine hydrochloride (17.9 g) was removed by filtration. The precipitate of the crude compound B hydrochloride (4.6 g) was further removed by adding 150 ml of ether. The mother liquor thus obtained was concentrated to dryness in vacuo and the brownish powder (34.1 g) was dissolved in the mixture of *n*-butanol, acetic acid and water (4 : 2 : 1 V). The solution was introduced into the top of the column (6.5 cm in diameter), which was packed 400 g of cellulose powder (Whatman), and developed chromatographically with the mixture of *n*-butanol, acetic acid and water (4 : 2 : 1 V). The initial effluent (2 l) from the column was discarded and the next fraction (600 ml) was shaken with 100 ml of water and 100 ml of ether. The lower layer (120 ml) was separated, washed with 50 ml of ether and was concentrated to dryness. The brownish powder (6.4 g) containing compound C hydrochloride was dissolved in methanol, mixed with ethanol and concentrated under reduced pressure. This procedure was repeated until methanol was replaced with ethanol. To the ethanol solution of compound C hydrochloride, excess amount of triethylamine was added, and resulting crystals (free base) were separated by filtration and washed with chloroform. Recrystallization from ethanol yielded 3 g. Twice recrystallization gave m.p. 160~162°. $[\alpha]_D^{17}$+

136° (c, 0.86 in water).

Anal. Calcd. for $C_6H_{12}NO_4(OCH_3)$: C, 43.5; H, 7.8; N, 7.3; OCH_3, 16.0; eq. wt. 193.

Found: C, 43.15; H, 7.3; N, 7.3; OCH_3, 15.8; eq. wt. 190.

The pKa′ of this compound was 7.7 (potentiometric titration).

Compound C N-acetate—A solution containing 300 mg of compound C free base in 7.5 ml of water and 0.75 ml of methanol was stirred for 4 hr. at 0~5° with 9 ml of Dowex-1 (carbonate form) and 0.3 ml of acetic anhydride. After stirring further for 1 hr. at room temperature, the mixture was filtered and the filtrate and washings were passed through a column containing 1.5 ml of Amberlite IR-120 (acid form). The effluent and washings were heated to boiling and the solution was concentrated to dryness in vacuo below 55°. The residue was dissolved in the mixture of methyl acetate and methanol (ca. 11 : 1 V) at 70° and then cooled, forming needle crystals. Yield, 180 mg. Recrystallized from the same solvents. m.p. 176~178°. $[\alpha]_D^{15}$ +158° (c, 0.40 in water).

Anal. Calcd. for $C_6H_{11}O_4N(OCH_3)(CH_3CO)$: C, 45.9; H, 7.2; N, 5.95.

Found: C, 45.7; H, 7.1; N, 5.9.

Periodate oxidation of compound C and its N-acetyl derivative—Periodate oxidations were done in the buffer of pH 4.7 as described under "Periodate oxidation of compound B hydrochloride and its N-acetyl derivative". Compound C free base consumed two moles of periodic acid within 5 hr. and the reaction was essentially completed. Compound C N-acetate was not attacked by periodic acid. Periodate oxidation of compound C yielded no formaldehyde.

Color reactions of compound C—Compound C was converted to demethyl derivative by boiling in 2 N hydrochloric acid for 7 hr. The color reactions of compound C and demethyl-C were tested.

	Compound C	Demethyl compound C
Red tetrazolium	−	+
Elson-Morgan	−	+
Molisch	−	±
Anhydrosugar	−	+

The intensities and absorption curves of anhydrosugar reaction and Elson-Morgan reaction of glucosamine (2-amino-2-desoxy-glucose) and demethyl compound C were compared.

Anhydrosugar reaction (Indole-HCl reaction): This reaction was done according to the description of Zacharias Dische[37]. Glucosamine and demethyl C gave the almost identical absorption curves (maxima at 492 mμ), and their intensities were not so much different. Elson-Morgan reaction[38]: N-acetyl compound C was heated in 2 N HCl at 100° for 2 hr. and the solution was neutralized with 2 N NaOH. N-Acetyl-α-methyl-2-glucosaminide was also hydrolyzed under the same condition. The Elson-Morgan reagents were added and after 30 minutes both amino sugars gave the almost identical absorption curves. But, after standing for 24 hr. at room temperature, the color of the former faded and almost transparent at 500~600mμ, while the absorption intensity of the latter (glucosamine) at 530 mμ was stronger than that of the day before.

Tetraacetate of compound C—Nine hundred mg of crude N-acetyl compound C was refluxed with 3.0 g of fused sodium acetate in 15 ml of acetic anhydride in an oil bath for 7 minutes. After removal of acetic anhydride in vacuo, the residue was taken to chloroform and evaporated. The residue was treated with acetone and crystals were separated. Recrystallization from acetone gave plate crystals. Yield, 885 mg. m.p. 176~177°. $[\alpha]_D^{28}$ +109° (c, 0.423 in chloroform).

Anal. Calcd. for $C_{15}H_{23}NO_9$: C, 49.8; H, 6.4; N, 3.9.

Found: C, 49.9; H, 6.4; N, 3.9.

The melting point, the optical rotation and infrared spectrum of this compound were identical to those of the synthetic methyl-3-amino-3-deoxy-α-D-glucopyranoside tetraacetate, described below and admixture showed m.p. 176~177°.

Strontium D′-methoxy-D-hydroxymethyl diglycolate from compound C.—Compound C free base (300 mg) and sodium bicarbonate (200 mg) were dissolved in 0.54 M periodic acid solution (6 ml). The solution, after being diluted with water to 15 ml, was kept at 20~25° for about 7 hr. The solution was mixed with ca. 700 mg of barium chloride and 200 mg of barium carbonate and, after stirring, the precipitate was removed by filtration. The filtrate was concentrated under reduced pressure to dryness and was dissolved in ethanol. After removing ethanol-insoluble material, the solution was concentrated to a colorless syrup. Two-hundred mg of this syrup (dialdehyde) was dissolved in 20 ml of water and 3 g of strontium carbonate and 0.4 ml of bromine were added. The mixture was kept in the dark at room temperature for about 18 hr. with frequent

37) Zachrias Dische, *Methods of Biochemical Analysis*, **2**, 353 (Interscience Pub. New York).

38) Richard J. Winzler: *ibid.*, **2**, 293

shaking. Excess bromine and excess strontium carbonate were removed. The filtrate was shaken with silver carbonate (ca. 2 g), and the silver was removed as sulfide, and excess hydrogen sulfide was expelled by aeration. The solution was concentrated by mixing with ethanol to a small volume, resulting needle crystals. Yield, 25 mg. Recrystallized from water-ethanol. $[\alpha]_D^{20}$ −40° (in water).

Anal. Calcd. for $C_6H_8O_7Sr \cdot 2H_2O$: C, 22.8; H, 3.8.
Found: C, 22.5; H, 3.4.

For comparison, α-D-methylglucoside (300 mg) was also oxidized according to E. L. Jackson and C. S. Hudson[17], giving 170 mg of strontium D'-methoxy-D-hydroxymethyl diglycolate. Both strontium salts of the diacids showed the identical infrared absorption spectrum.

Phenylosazone derivative from compound C—A solution of 1.4 g of compound C free base in 45 ml of 2 N hydrochloric acid was refluxed for 7 hr. To this reaction mixture, a small amount of *p*-phenetidine, 2.0 g of phenylhydrazine, 1 ml of acetic acid and 14 g of sodium acetate were added. The mixture was heated for 1.5 hr. on boiling water bath and then cooled. The crystals formed were collected and recrystallized from ethanol-ether. Yield, 200 mg. m.p. 222~222.5°, $[\alpha]_D^{25}$ −82°→−72° in 17 hr. (c, 0.401 in pyridine-50% methanol (4 : 6)).

Anal. Calcd. for $C_{18}H_{23}O_3N_5 \cdot HCl$: C, 54.9; H, 6.1; N, 17.7.
Found: C, 55.3; H, 6.2; N, 17.3.

This compound gave a positive ninhydrin test and a single spot in circular paper chromatography[39]. (The average R_F value 0.07 in solvent system: toluene, ethanol, H_2O 270 : 30.1). Its ultraviolet spectrum showed typical sugar phenylosazone[40]. (λ_{max}. 254 mμ, 310 mμ, 400 mμ (log ε : 4.105) in 95% ethanol).

Phenylflavazol derivative from compound C—Five hundred milligram of compound C was boiled in 20 ml of 2N hydrochloric acid for 8.5 hr. The solution was evaporated to dryness and dissolved in 20 ml of water. Paperchromatography of this solution showed chiefly demethyl compound C, but a small amount of compound C was detected. To the above solution, 0.3 g of *o*-phenylenediamine, 1.4 g of phenylhydrazine and 1.0 g of acetic acid were added, and the mixture was heated at 100° in a sealed tube under carbon dioxide for 24 hr. On cooling, a dark red resinous product was precipitated, which after washing with water, was extracted with ethanol and the extract evaporated to dryness. For purification, the crude product was dissolved in dioxane and chromatographed on an acid washed alumina. Dark colored impurities were removed from the column by elution with dioxane, and subsequent elution with dioxane containing 2% methanol and concentration of the eluate yielded yellow needle crystalls of phenylflavazol[41]. Recrystallization from dioxane yielded 51mg, m.p. 213~215.

Anal. Calcd. for $C_{18}H_{16}O_3N_4$: C, 64.25; H, 4.8; N, 16.7.
Found: C, 64.7; H, 4.2; N, 16.7.

The mixed melting point with the authentic D-erythro-trihydroxypropyl-1-phenylflavazol (m.p. 213~216°) from D-glucose was 213~216°.

Acetylation of this compound (24 mg) with acetic anhydride (360 mg) at 100° for one hr. and crystallization from ethanol gave triacetylphenylflavazol (21 mg), m.p. 120.5~123°, $[\alpha]_D^{26}$ +64.0° (c, 0.40 in chloroform).

Anal. Calcd. for $C_{24}H_{22}O_6N_4$: C, 62.3; H, 4.8; N, 12.1.
Found: C, 61.8; H, 4.5; N, 12.4.

The mixed melting point of this compound with synthetic D-erythrotrihydroxypropyl-1-phenylflavazol triacetate, was 120~123°.

Synthesis of methyl 3-amino-3-deoxy-α-D-glucopyranoside tetraacetate[19]—A solution of 7.3 g of methyl-α-D-glucopyranoside 2, 4, 6-triacetyl-3-*p*-toluensulfonate (syrup: $[\alpha]_D^{28}$: +50°) in 36 ml of methanol saturated with ammonia at 5°, was heated in a sealed tube at 150° for 30 hr. The solution was evaporated to syrup, which was taken to alcohol and ammonium *p*-toluenesulfonate was removed by addition of ether. The syrupy residue obtained on evaporation of the filtrate was boiled for 7 minutes with acetic anhydride (30 ml) and sodium acetate (6.0 g), and evaporated again to a syrup which was dissolved in chloroform, treated with Darco G-60 and concentrated in vacuo to dryness. The residue was crystallized from acetone. Recrystallization from the same solvent gave methyl 3-amino-3-deoxy-α-D-glucopyranoside tetraacetate (610 mg), m.p. 177°, $[\alpha]_D^{29}$ +107° (c, 0.44 in chloroform).

Anal. Calcd. for $C_{15}H_{23}O_9N$: C, 49.8; H, 6.4; N, 3.9.
Found: C, 49.7; H, 6.4; N, 3.9.

39) D. C. Barry and P.W.D. Mitchell, *J. Chem. Soc.*, **1954**, 4023. In the same solvent system D-glucose phenylosazone showed the average R_H value 0.41.

40) V. D. Barry, J. E. McCormick and P.W.D. Mitchell, *J. Chem. Soc.*, **1955**, 222.

41) The final methanol eluate of this column gave a small amount of phenylosazone which identified as phenylosazone hydrochloride of demethyl C by ultraviolet and infrared spectra. The separation of 1-phenylflavazol derivative from attended by-product (phenylosazone derivative) was very troublesome by Ohle's method (H. Ohle and J. J. Kruyff, *Ber.*, **77**, 512 (1944)), but was simply achieved by the above meantioned alumina column chromatography.

Deamination of kanamycin—One g of Kanamycin monosulfate was dissolved in 8 ml of water and after the pH was adjusted to 5.0 with hydrochloric acid, 770 mg of sodium nitrite was added. The solution was allowed to stand for 2.5 hr. at room temperature (during this time, the rotation changed from $[\alpha]_D^{18}+108°$ to $[\alpha]_D^{18}+99°$). The solution colored to reddish browen but the reducing power was almost negative. When 0.6 ml of acetic acid was added to this solution, gas evolved and rotation changed from $[\alpha]_D^{18}+99°$ to a final value of $[\alpha]_D^{18}+33°$, and reducing power was appeared. The reaction mixture was neutrallized with barium hydroxide and the filtrate was lyophilized. Paperchromatography (solvent system: *n*-butanol, acetic acid, water 4: 1: 2.5 V) showed two spots at R_F 0.45 and 0.27, developed with aniline hydrogen phthalate. The substance of R_F 0.45 seemed to be anhydrohexose, the R_F value of 0.27 was coincided with that of D-glucose. The above reaction products were dissolved in 15 ml of water, and 1 g of *p*-phenetidine and a small amount of acetic acid were added. After heating for 15 minutes in boiling water, the reaction mixture was decanted to remove resinous product, and supernatant solution was reacted with phenylhydrazine and acetic acid for 20 minutes in boiling water. The black brown precipitate, which formed on cooling, was dissolved in methanol-water and the solution allowed to stand at room temperature for several days. The separated yellow crystals were recrystallized from 95% ethanol. Yield, 77 mg. m.p. 206~208° (des.)

$[\alpha]_D^{24}-90°\rightarrow-84°$ after 24 hours (c, 0.45 in dimethyl sulfoxide).

Anal. Calcd. for $C_{18}H_{24}O_4N_4$: C, 60.3; H, 6.2; N, 15.6.

Found: C, 60.7; H, 6.1; N, 15.6.

The authentic sample of D-glucose phenylosazone showed m.p. 202~205°, $[\alpha]_D^{24}-94°\rightarrow-86°$ in 24 hours (c, 0.38 in dimethyl sulfoxide). The infrared spectra of these two compounds were identical.

D-Glucose thus formed from kanamycin was well understood to be derived from compound B.

Treatment of N-acetylkanamycin with sodium nitrite in the presence of acetic acid, did not hydrolyze the glycosidic bond.

Quantitative periodate oxidation of N-acetylkanamycin and kanamycin—Quantitative periodate oxidation were done in the buffer of pH 4.7 as described under "Periodate oxidation of compound B hydrochloride and its N-acetyl derivative".

N-acetylkanamycin;

Consumed periodate per mole

Time (hours)		
19	43	96
2.0	2.0	2.0 moles

Kamamycin free base:

Consumed periodate per mole

Time (hours)			
5	24	48	95
4.4	5.2	6.3	7.4 moles

But in 0.02 M periodic acid solution (pH 1.8), the periodate oxidation of kanamycin free base indicated the almost theoretical results; to an equeous solution containing 22 mg of kanamycin free base, 2.0 ml of of 0.27 M periodic acid solution was added, and was immediately diluted to a total volume of 25 ml.

Aliquots were withdrawn for titration.

Consumed periodic acid per mole

Time (hours)		
5	28	48
4.3	4.4	4.5 moles

Isolation of formic acid by periodate oxidation of N-acethylkanamycin—In 20 ml of water, 1.306 g (2.0 millimole) of N-acetylkanamycin and 1.115 g (4.2 millimole) of sodium metaperiodate were dissolved and allowed to stand at room temperature for 20 hr. The solution was concentrated to dryness in vacuo and the distillate condensed in a dry ice-ethanol trap. The distillate was neutralized with sodium hydroxide (0.9 millieq. was required) and concentrated to dryness to yield 99 mg of solid sodium formate which was further identified as its S-benzylthiouronium salt (m.p. 144~146°).

Also, in the periodate oxidation according to Morrison's method[42] from one mole of N-acetylkanamycin, one mole of formic acid was determined by titration.

Isolation of deoxystreptamine by periodate oxidation of N-acetylkanamycin — N-acetylkanamycin (0.82 g, 1.26 millimole) was dissolved in 250 ml of 0.02 M periodic acid solution (5.00 millimole) and left standing at 5~10° for 42 hr. The reaction mixture (225 ml) was neutrallized with barium hydroxide, precipitates removed and concentrated in vacuo to dryness. The resulting white powder (0.73 g) was extracted with 5 ml of methanol which was evaporated in vacuo (0.71 g) and redissolved in 10 ml of water and treated with sodium borohydride in the usual way[43]. The reduced alkaline solution was neutrallized with acetic acid and evaporated in vacuo (0.98 g) and extracted

42) M. Morrison, A. C. Kuyper and J. M. Orten, *J. Am. Chem. Soc.*, **75**, 1502 (1953).

43) I. J. Goldstein, J. K. Hamilton, R. Montogomery and F. Smith, *J. Am. Chem. Soc.* **79**, 6469 (1957).

with 5 ml of methanol. The extract was evaporated to dryness in vacuo (0.86 g) and was refluxed in 8 ml of methanolic hydrogen chloride (10 N) for 10 hr. After the methanolysis, white precipitate (0.28 g) which showed R_F 0.15~0.17 by paper-chromatography (*n*-butanol, acetic acid, water (4 : 1 : 2.5), ninhydrine), was obtained. Eighty mg of this precipitate was heated in 0.5 ml of water with picric acid (100 mg in 2 ml of water) on boiling water bath, resulting 108 mg of the yellow crystalline picrate which recrystallized from 80% ethanol gave, m.p. 254~262° (dec.).

Methylation of N-acetylkanamycin and isolation of monomethyl deoxystreptamine — N-acetylkanamycin (4 g) was methylated with dimethyl sulfate and alkali by the analogous method of J. Fried and H.E. Stavely[44]. Two grams of crude methylated product was obtained. Methoxyl content, 27.6%. This product gave positive ninhydrin reaction. Paper chromatogram of this product gave two ninhydrin-positive spots, R_F 0.50 (weak) and 0.62 (solvent : *n*-butanol, acetic acid, water). So it was re-N-acetylated as described under "N-Acetyl derivative of compound C" (the methoxyl content of re-N-acetylated product, 24.6%) and then further methylated with methyliodide and silver oxide by the analogous procedure of Maurice Stacey[45].

The amorphous methylated product, thus obtained, showed methoxyl content 27.8.

Calcd. for $C_{18}H_{21}O_4(OCH_3)_7(NH\cdot COCH_3)_4$: OCH_3, 28.9.

This compound (1.5 g) was hydrolyzed by boiling in 2N HCl for 5.5 hr. and concentrated to a small volume in vacuo. To this solution, ethanol was added, yielding 140 mg of fine crystals, which recrystallized from water-ethnal, decomposed partially at 200~210°, and completely at 240~250°.

$[\alpha]_D^{28}$ 0 (c, 1.4 in water). R_F value 0.16 (solvent : *n*-butanol, ethanol, water 4 : 1 : 5 V) and 0.31 (solvent : *n*-butanol, acetic acid, water 4 : 1 : 2.5 V).

Anal. Calcd. for $C_7H_{16}N_2O_3\cdot 2HCl$: C, 33.75; H, 7.3; N, 11.2; OCH_3, 12.5.

Found : C, 33.1; H, 7.1; N, 11.0; OCH_3, 12.0.

This comound (40 mg) was acetylated by refluxing for ten minutes in a mixture of acetic anhydride and pyridine. The crude tetraacetyl derivative was dissolved in 4 ml of water and a 2 ml portion of Amberlite IR 410 (OH) resin was added and the mixture was kept overnight with shaking. After removal of the resin by filtration, the filtrate was concentrated in vacuo, by mixing with ethanol, yielding crystals of mono-O-methyl-N,N'-diacetyl deoxystreptamine. This was recrystallized by dissolving in a small amount of water, and by concentrating with ethanol. Yield 12 mg.,

Anal. Calcd. for $C_6H_7(OH)_2(OCH_3)(NHCOCH_3)_2$: C, 50.8; H, 7.7; N, 10.8.

Found : C, 50.0; H, 7.6; N, 10.9.

O-α-D-3-amino-3-deoxy-glucopyranosyl-2-deoxystreptamine (Compound F)—Ten grams of kanamycin base was hydrolyzed partially by standing in 100 ml of 6 N HCl at3 7°C for 11 days (remaining activity : 1%). The hydrolyzates were adjusted to pH 5 with Amberlite IR 4B (carbonate from) and was concentrated to dryness in vacuo and the brownish powder (11.7 g) was dissolved in a mixture of *n*-butanol, acetic acid, and water (4 : 2 : 1). The solution was introduced into the top of the column (2.9 cm in diameter), which was packed 80 g of cellulose powder (Whatman), and developed chromatographically with the mixture of *n*-butanol, acetic acid and water (4 : 2 : 1). The initial effluent (3,400 ml) from the column (containing 3-amino-3-deoxy-D-glucose and 6-amino-6-deoxy-D-glucose) was discarded and 1,200 ml of the next fraction, which showed one spot of R_F 0.13~0.18 by paperchromatography (*n*-butanol, acetic acid, water (4 : 1 : 2.5), ninhydrin), was shaken with water and ether. The lower layer was separated and washed with ether and was concentrated to dryness in vacuo, resulting 1.5 g of white amorphous powder. This powder was dissolved in 20 ml of methanol-water (1 : 1) and added with ethanol.

By standing in refrigerator, 710 mg of O-α-D-3-amino-3-deoxy-glucopyranosyl-2-deoxystreptamine trihydrochloride crystallized out. Recrystallized sample from a mixture of water, methanol and ethanol gave m.p. 244~6°C (dec.), $[\alpha]_D^{30}$+69.9° (c, 0.5, in water).

Anal. Calcd. for $C_{12}H_{25}N_3O_7\cdot 3HCl$: C, 33.3; H, 6.5; N, 9.7; Cl, 24.6.

Found : C, 33.45 : H, 6.2; N, 9.6; Cl, 24.8.

It gave negative reducing sugar test. The methanolysis of the compound F hydrochloride showed two spots of which R_F values were coincided with those of deoxystreptamine (0.23) and methyl-3-amino-3-deoxy-α-D-glucopyranoside (0.48) by paperchromatography (*n*-butanol, acetic acid, water (4 : 1 : 2.5 ninhydrin). The crystalline free base of compound F was obtained from the hydrochloride by treating with Amberlite IRA 400 (OH) and recrystallized from the mixture of methanol and ethanol. m.p. 238~242°C (dec.).

The crystalline N-acetyl derivative of compound F

44) J. Fried and H. E. Stavely, *J. Am. Chem. Soc.*, **74**, 5461 (1952).

45) Maurice Stacey, *J. Chem. Soc.*, **1944**, 272.

was prepared on treatment with acetic anhydride in methanol from the crystalline free base. m.p. 176~181°C (dec.).

Anal. Aalcd. for $C_{12}H_{22}N_3O_7 \cdot 3(CH_3CO)$: C, 48.1; H, 6.95; N, 9.35.

Found: C, 47.7; H, 7.2; N, 8.9.

By the periodate oxidation in 0.02 M periodic acid solution at 5~10°C for 24 hr. the hydrochloride of compound F consumed 4.0 moles of periodic acid and the free base consumed 3.8 moles, while N-acety derivative consumed 1.0 mole of periodic acid.

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Studies on the Metabolism of D-Amino Acid in Microorganisms

Part VII. Possible Involvement of Cell Permeability in the Metabolism of L-Glutamic Acid by *Aerobacter aerogenes* Strain A

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Several experiments were made in order to clarify the reason why *Aerobacter aerogenes* strain A can grow well on D-glutamate-K_2HPO_4 medium, while it can not grow on L-glutamate medium at neutral pH value. The permeabilities of L- and D-glutamic acids to the bacterial cell membrane seem to play some important role on this phenomenon judging from several experimental results on growth tests and enzymatic studies.

INTRODUCTION

In the previous papers[1,2], it was described that several strains belonging to *Aerobacter aerogenes* could grow well on D-glutamate-K_2HPO_4 medium, while it showed only scanty growth on L-glutamate medium at neutral pH value. The resting cells of the bacterium oxidized D-glutamic acid most rapidly at about pH 8.0, while L-glutamic acid was oxidized

1) K. Izaki, H. Takahashi and K. Sakaguchi, This Bulletin, **19**, 233-239 (1955).
2) K. Izaki, This Bulletin, **22**, 78-84 (1958).

much faster at an acidic condition than a neutral pH value. Hence, the author assumed that the permeabilities of D- and L-glutamic acids to the baterial cell membrane might be involved in the above phenomena. In this paper, the author has made further studies on this point and obtained several experimental results that favored this assumption.

METHODS AND MATERIALS

1. Cultural condition. The bacterium was grown aerobically in Monod's shaking tube by the use of a Monod's shaking machine at 30°C and, the growth rate was determined by a nepherometer as mentioned in the previous report[2]. Various media used will be described under the paragraph of Experimental results.

2. Materials. D-, L and DL-glutamic acids and α-ketoglutaric acid were supplied through the courtesy of of the Ajinomoto Co., Ltd. D-Alanine, D-aspartic acid and L-alanine were purchased from the California Foundation for Biochemical Research, U.S.A.. Other DL-amino acids used here were products of General Biochemical Inc., U.S.A.. DPN* and TPN* were the products of the California Foundation for Biochemical Research, U.S.A..

3. Methods for determining metabolic activity. Oxygen uptake was measured using a Warburg respirometer at 30°C. L-Glutamic acid was estimated by L-glutamic acid decarboxylase obtained from *E. coli*[3]. The reduction of TPN or DPN was measured at 340 mμ using a Hitachi spectrophotometer (EPU-2A).

EXPERIMENTAL RESULTS

I. Effects of various conditions on the growth of *Aerobacter aerogenes* strain A.

1) Effect of amino acid. *Aerobacter aerogenes* strain A was grown under an aerobic condition on various amino acids-mineral media and growth rates were measured. The medium had the following composition; D- or L-amino acid 0.5%, K_2HPO_4 0.2%, NaCl 0.5% and $MgSO_47H_2O$, 0.02%.

The medium was adjusted to pH 7.2. The results are given in Fig. 1. The bacterium grew well on D-glutamate, DL-alanine and L-alanine media, whereas, growths on L-glutamate and L-aspartate media were very poor and no growth was observed on D-aspartate and both isomers of methionine media. D-Alanine seemed to be somewhat superior to L-isomer as a growth medium. This result was also confirmed by another experiment using D-alanine in place of DL-alanine. The superiority of D-amino acid to L-isomer as a growth medium was however, only clearly demonstrated in the case of glutamic acid as shown in Fig. 1. Inability of growth on D-aspartate medium may be explained by the absence of D-aspartate oxidizing system in this bacterium as reported previously[2].

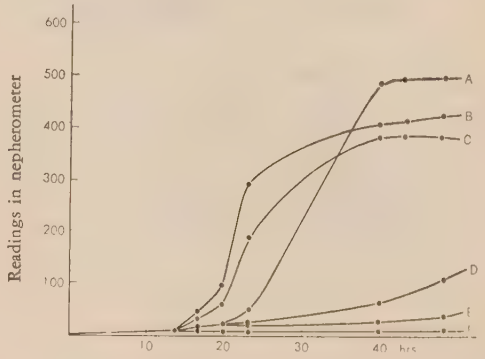

FIG. 1. Effect of amino Acid on Growth of *Aerobacter aerogenes* A. (Amino acid-mineral medium)

A. D-glutamate
B. DL-Alanine
C. L-Alanine
D. L-Aspartate
E. L-Glutamate
F. D-Aspartate
L-Methionine
DL-Methionine

2) Effect of pH upon the growth on L-glutamate medium. L-Glutamate-mineral medium containing 0.5% L-glutamic acid, 0.2% K_2HPO_4, 0.5% NaCl, and 0.02% $MgSO_47H_2O$ was used as a basal medium. The pH of media were adjusted to 3.4, 4.4, 5.4 and 7.2, respectively by the addition of dilute hydrochloric acid or sodium hydroxide solution. The effect of pH is clearly shown in Fig. 2. The bacterium could grow well on the L-glutamate-medium at acidic pH value. The bacterium, however, could not grow at such a low pH level as 3.4. Addition of L-pyroglutamic acid to the basal medium had not any promoting effect upon bacterial growth.

3) Effect of L-glutamic acid concentration. As illustrated in Fig. 3, remarkable difference of bacterial growth was not observed at the concentration of L-glutamic acid ranging from 0.5 to 2.0% though the high concentration somewhat favored growth.

* DPN: Diphosphopyridine nucleotide
* TPN: Triphosphopyridine nucleotide.

3) W. W. Umbrit and I. C. Gunsalus, *J. Biol. Chem.*, **159** 333 (1945).

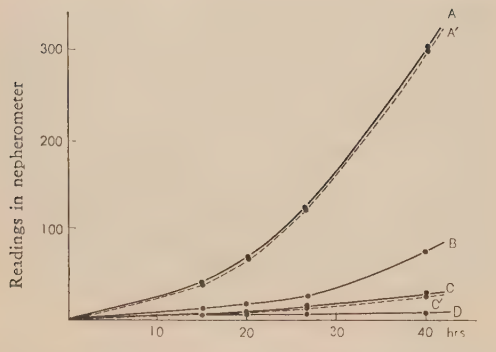

FIG. 2. Effect of pH on Bacterial Growth. (L-Glutamate-mineral medium)

A. pH 4.4
A'. pH 4.4+0.02% L-pyroglutamic acid
B. pH 5.4
C. pH 7.2
C'. pH 7.2+0.02% L-pyroglutamic acid
D. pH 3.4

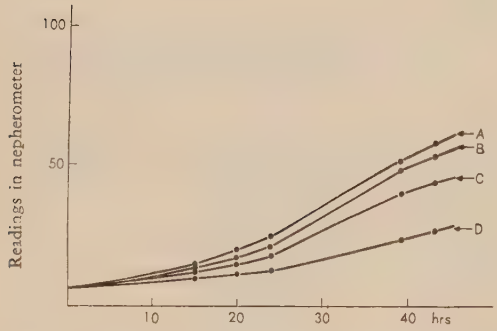

FIG. 3. Effect of L-Glutamate Concentration on Bacterial Growth. (L-Glutamate-mineral medium)

A	L-Glutamate	2.0%
B	〃	1.5%
C	〃	1.0%
D	〃	0.5%

4) Effects of glucose, citrate, D-glutamate, ammonium chloride and vitamins. Various amounts of energy sources such as glucose, citrate and D-glutamate were added to the L-glutamate basal medium and growth rates were measured. All media were adjusted to pH 7.2. The effects of nitrogen source (ammonium chloride) and vitamin mixture, which was composed of B_1, B_2, B_6 (pyridoxal, pyridoxine), nicotinic acid, pantothenic acid, *p*-amino benzoic acid, and folic acid, were also examined. The results are presented in Fig. 4 and Fig. 5. As shown, all three energy sources were effective for bacterial growth at the concentration of 0.1% and of these, glucose was most effective. It was also observed

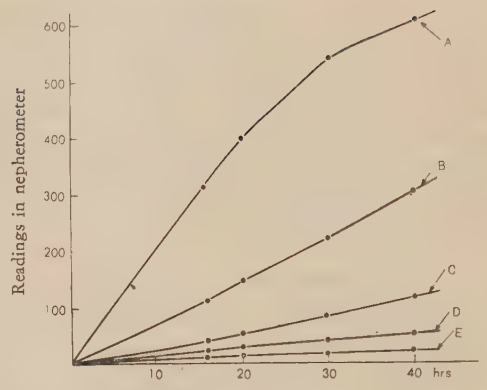

FIG. 4. Effects of Glucose, Citrate, Ammonium Chloride and Vitamin Mixture on Bacterial Growth. (L-Glutamate-mineral medium)

Addition
A. 0.1% glucose
B. 0.1% citrate
C. 0.01% glucose
D. 0.01% citrate
E. 0.1% ammonium chloride or vitamin mixture※, or none
※ The concentration of each component was μg per ml

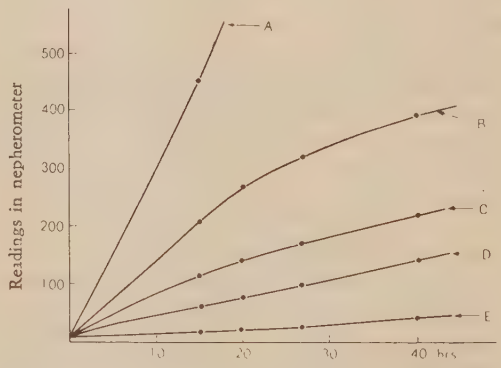

FIG. 5. Effect of D-Glutamate Concentration on Bacterial Growth. (L-Glutamate-mineral medium)

A	D-Glutamate	0.5%	added
B	〃	0.1%	〃
C	〃	0.05%	〃
D	〃	0.02%	〃
E	〃	no	〃

that the effects of these energy sources were promoted in proportion to the increase of thier concentrations. The effect of glucose was not considered to be indirect, as was due to reduced pH of the medium caused by organic acid formation from the sugar, since the reduction of pH of the medium was not observed during

cultivation. The addition of ammonium chloride or vitamin mixture to the basal medium had not any promoting effect upon bacterial growth.

II. Effect of pH on the metabolic activity of *Aerobocter aerogenes* strain A towards L-glutamic acid.

The bacterial metabolic activity towards L-glutamic acid was determined by the rate of consumption of either L-glutamic acid or oxygen. The bacterium was aerobically incubated with L-glutamic acid and phosphate buffer (pH 7.2) in Monod's test tube using Monod's shaking machine at appropriate intervals and the consumption of L-glutamic acid was determined by the usual enzymatic method. During incubation, growth of the bacterium was not observed. The rate of oxygen uptake was measured by a Warburg respirometer. Both the resting cells and the freeze-thawing treated cells were used; these cells being obtained by the following procedures. *Aerobacter aerogenes* A was grown aerobically in a nutrient broth containing meat extract 1%, polypepton 1%, NaCl 0.5% at a neutral pH condition from fifteen to sixteen hours. The cells were harvested by centrifugation and washed twice with distilled water and a portion was resuspended in distilled water (resting cell suspension); another portion in a state of paste was freezed and thawed repeatedly (about ten times) by puting it in and taking it out from a deep freezer (freeze-thawing treated cells). The results and experimental conditions are shown in Fig. 6 and Fig. 7. The weakly acidic condition (about pH 5.4) was again found favorable for the oxidation of L-glutamic acid by the resting cells. In accordance with this result, the rate of L-glutamic acid consumption by the resting cells was maximum at pH 5.4. On the other hand, the rate of L-glutamic acid consumption by freeze-thawing treated cells was maximum at neutral or weakly basic pH values (pH 7.0~8.0). From the above results it appears that the permeability of L-glutamic acid to the bacterial cell is involved in the metabolism of L-glutamic acid by the intact cells.

III. Enzymes responsible for L-glutamic acid degradation.

The presences of various enzymes, such as L-glutamic dehydrogenase, L-glutamic acid- other amino acid transaminase, glutamic racemase and L-glutamic decarboxylase, which were responsible for L-glutamate degradation were investigated. Of these enzymes, L-glutamic dehydrogenase, L-glutamic- other amino acids transaminases and aspartase were clearly detected in the cells of *Aerobacter aerogenes* A. The presence of glutamic racemase and L-glutamic decarboxylase, however, could not be demonstrated by any means.

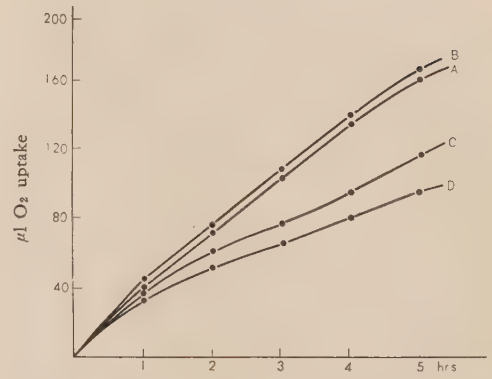

FIG. 6. Effect of pH on the L-Glutamate Oxidation by Resting Cells of *Aerobacter aerogenes* A.

A pH 4.4
B 5.4
C 7.2
D 8.0

Each vessel contains resting cell suspension 0.5 ml (1.0 mg dry weight), M/1° phosphate buffer 1.0 ml and 100 μM of L-glutamate in a total volume of 2.0 ml. 0.2 ml of 20% KOH is placed in the center well. The values are corrected by subtracting endogenous values.

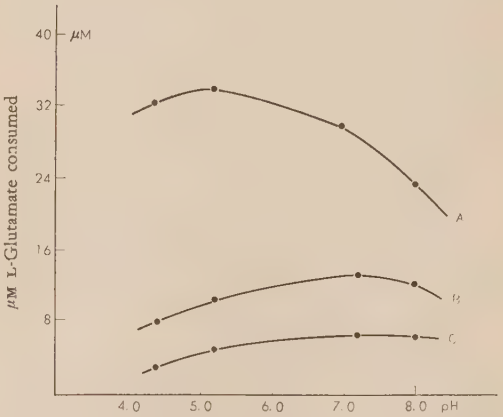

FIG. 7. Effect of pH on Consumption of L-Glutamic Acid by Resting Cells or Freeze-thawing Treated Cells of *Aerobactor aerogenes* A.

A Resting cells; two hours incubation
B Freeze-thawing treated cells; four hours incubation
C Freeze-thawing treated cells; two hours incubation

Each Monod's tube contains resting cell suspension or freeze-thawing treated cell suspension 1.0 ml (20 mg dry weight), M/10 phosphate buffer pH 7.2, 2.0 ml and 50 μM of L-glutamate in a total volume of 5.0 ml. The incubation times were as indicated in Fig. 7. temperature: 30°C.

1) L-Glutamic dehydrogenase. *Aerobactor aerogenes* A was grown aerobically from fifteen to sixteen hours in the nutrient broth at neutral pH value and the cells were harvested by centrifugation and washed twice with distilled water and finally suspended in a phosphate buffer (pH 7.2) to give a final concentration of approximately 10 mg dry weight per ml. The thick cell suspension thus obtained was sonicated at 9~10 kilocycle/sec. for 20 minutes in the cold by the use of a sonic occillator and the entire sonicates were centrifuged at 13000 g for 30 minutes to remove damaged and intact cells. The supernatant was dialyzed with stirring over night against M/50 phosphate buffer (pH 7.2) at 2°C. The dialyzed supernatant was used as a enzyme preparation. The activity of L-glutamic dehydrogenase was determined by measuring TPN or DPN reduction at 340 mμ at 15°C. The result is shown in Fig. 8.

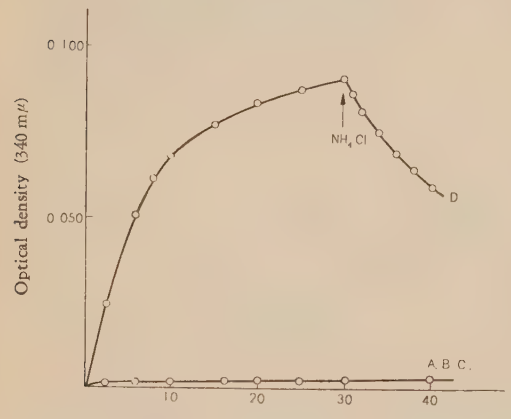

FIG. 8. Requirement of TPN for L-Glutamic Dehydrogenase.

Composition of reaction mixture.

	A	B	C	D
Dialyzed extract	1.0 ml	1.0ml	1.0 ml	1.0 ml
M/10 L-Glutamate	—	—	0.5	0.5
DPN (μM/ml)	0.4	—	0.4	—
TPN (μM/ml)	—	0.4	—	0.4
M/5 Phosphate buffer pH 7.2	0.5	0.5	0.5	0.5
H_2O	1.1	1.1	0.6	0.6

It is evident from this result that L-glutamic dehydrogenase requires TPN as a coenzyme. L-Glutamic dehydrogenase of this bacterium was active at neutral and weak basic pH values.

2) Transaminase. Resting cells and lyophilized cells were used as enzyme preparations. Resting cells were prepared as described in experimental results II, and the paste of the resting cells were lyophilized and used as lyophilized cells. Transaminases were detected only when lyophilized cells were used. Experimental conditions were as follows: each Thunberg tube contained 0.5 ml of lyophilized cell suspension (10 mg dry weight), 0.5 ml of M/10 α-ketoglutaric acid, 0.5 ml of M/10 various amino acids and 1.0 ml of M/10 phosphate buffer pH 7.2. Incubation was conducted under an anaerobic condition at 30°C for two hours. After incubation, the reaction was stopped by heating and the cells were removed by centrifugation. The supernatant was analyzed by paperchromatography. Transamination reactions between α-ketoglutarate and the following amino acids, L-aspartate, DL-valine, DL-isoleucine and DL-methionine were detected. Among them, L-glutamate-L-aspartate transaminase was most active. L-Glutamate-L-alanine transaminase and D-amino acid transaminase between α-ketoglutarate and D-aspartate or D-alanine could not be detected. Aspartase was found again as described in the previous report[2]. All these transaminases and aspartase were active at a neutral pH value.

IV. Effect of pH upon bacterial growth on D-glutamate medium.

Effect of pH upon the growth of *Aerobacter aerogenes* A on D-glutamate medium was examined and compared to the case of L-isomer medium. Experimental conditions and culture medium were the same as described in Experimental results I. The bacterium could grow well on D-glutamate-K_2HPO_4 medium at a neutral pH value and even under a weak acidic condition as illustrated in Fig. 9. Similar growth curves were obtained in the case of *Aerobacter aerogenes* ATCC 9621, 8329 and 8308. The oxidative activities of both the resting cells and the cell-free extract of *Aerobacter aerogenes* A towards D-glutamic acid were highest at weak basic pH values (about pH 8.0), as reported previously[2]. These results were in marked contrast to the case of L-isomer.

DISCUSSION AND CONCLUSION

Various conditions which favored the growth of *Aerobacter aerogenes* strain A on L-glutamate-K_2HPO_4 medium were investigated. Reducing the initial pH of the medium to an acidic condition (about pH 4.4), or addition of energy source such as glucose or citrate at a neutral pH value was found to be effective, while addition of ammonium chloride or vitamin mixture had not any promoting effect on growth. Other experiments reveal that the rates of oxidation

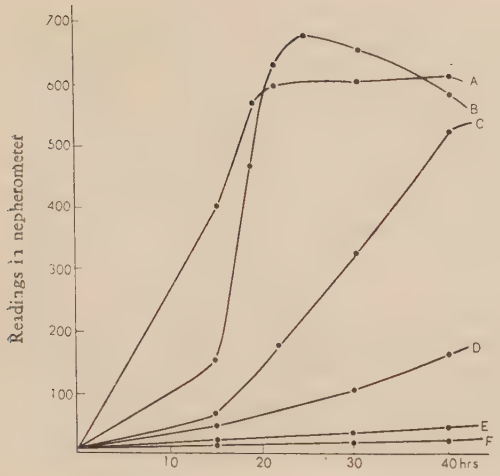

FIG. 9. Effect of pH on Bacterial Growth.
(Amino acid-mineral medium)

A	D-Glutamate	pH 7.2
B	〃	pH 4.4
C	L-Glutamate	pH 4.4
D	L-Aspartate	pH 4.4
E	L-Aspartate	pH 7.2
F	L-Glutamate	pH 7.2

and consumption of L-glutamic acid by the intact cells were greatest at a weak acidic condition (about pH 5.4). In contrast to this result, the optimum pH for L-glutamic acid consumption was shifted from acidic pH values to a neutral or basic pH values, when the freeze-thawing treated cells were used instead of the intact cells.

From enzymatic studies, L-glutamic dehydrogenase and L-glutamic- other amino acid transaminase, which were responsible for the metabolism of L-glutamic acid were found in the organism. These enzymes were active at neutral and weak basic pH values. From these results, it is natural to consider that the permeability of L-glutamic acid to the bacterial cell membrane has an important effect on the metabolism of L-glutamic acid by the intact cell of the bacterium. Decrease in pH was not observed when glucose or other energy sources were added to the L-glutamate-mineral medium, and therefore pH and energy source seem to exert some similar promoting effects on the penetration of L-glutamic acid into the cells by different mechanisms. In the case of D-glutamic acid, the bacterium could grow well on D-glutamate-K_2HPO_4 medium at a neutral pH value and even at a weak acidic condition. The metabolic activities of the intact cells and the cell-free extracts towards D-glutamic acid were also highest at about pH 8.0. These effects of pH are in marked contrast to those in the case of L-isomer. Therefore, the mechanism of D-glutamic acid penetration into the bacterial cell seems to be different from that of L-glutamate penetration. At all events, the permeabilities of L- and D-glutamic acids to the bacterial cell membrane seem to have a remarkable influence on the preferential utilization of D-glutamic acid to L-isomer at a neutral pH value. Whether D-glutamic acid is essential or not for this bacterium is a matter somewhat questionable. The bacterium could grow well on L-glutamate-K_2HPO_4 medium under acidic condition, and nevertheless, glutamic racemase, D-glutamic acid forming enzyme, could not be found in the bacterium. D-Glutamic acid, therefore, does not seem to be essential for the becterium.

Acknowledgement. The author wishes to express his sincere thanks to Emeritus Professor K. Sakaguchi and Professor K. Arima for their constant guidance throughout the course of this work.

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Studies on Dry Yeast. (IV)

Mechanism of Fermentation of Dry Bakers' Yeast

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The volume of carbon dioxide evolved in a shaking apparatus was measured at fixed intervals, and it was confirmed that the fermentation of dry yeast generally depends on three factors and that they are represented with an initial fermenting activity, a power which recovers fermenting activity, and a maximum fermenting activity. This expression of fermenting power was compared with usual expressions and it was proved to be more reasonable, particularly, for deciding the initial fermenting activity.

In the fresh bakers' yeast, uniform dispersion is easy and almost constant fermenting activity appears from the start of measurement[1], but, when yeast is dried and stored, an induction period[2], the Harden-Young effect[3], and difficult uniform dispersion of the yeast are notable. R. Willstätter and W. Steibelt stated that in order to determine fermenting activity of dry yeast it is necessary to shake the medium and examine the fermentation process at fixed intervals[1]. However, the modified Meissl's method mentioned in the section "Methods for examining the quality of bakers' yeasts"[4] was the only procedure which had usually been used for testing fermentation activity of bakers' yeast in Japan[5,6]. Even in application of another new method using a shaking apparatus[7], it is necessary to decide how the measured values are to be treated, particularly, in case of dry yeast.

Because it was mentioned in the previous report that, as the fermenting activity of dry yeast changes with the progress of fermentation, the fermenting power of dry yeast, i.e., the power which the yeast expends in causing fermentation, should not be expressed with one value determined at one period but with some values determined at several periods during the course of fermentation. From such reason, it is not reasonable in comparison of fermenting power of commercial bakers' yeast that the same method as that employed with fresh yeast is applicable with dry yeast. Much investigation has been made concerning the mechanism of fermentation by zymase or by dry beer yeast, particularly, about the so-called induction period, but the mechanism of fermentation by dry bakers' yeast has seldom been studied, although it is a recognized fact that the preparation of Lebedew juice or inhibition of toluene[8] is different when dry bakers' yeast in used than in case when dry beer yeast is used.

In the previous report, Q_{CO_2}(max), Q_{CO_2}(20′–40′), Q_{CO_2}(120′–140′), Q_{CO_2}(240′–260′) and t′*

1) R. Willstätter und W. Steibelt, *Z. für physiol. Chem.*, **115**, 211 (1921).
2) H. Katagiri and G. Yamagishi, *Biochem. J.*, **23**, 654 (1929).
3) O. Meyerhof and J. Westerberg, *J. Biol. Chem.*, **180**, 575 (1949).
4) Japan Yeast Research Soc., "Methods for Examining Quality of Bakers' Yeasts", Tokyo, 1949, p. 6.
5) R. Ishii, *J. Fermentation Technol.* (Japan), **34**, 102 (1956).
6) T. Sato, *J. Agr. Chem. Soc. Japan*, **31**, 491 (1957).
7) Assoc. of Japan Yeast Industry, "Methods for Examining Quality of Bakers' Yeasts", Tokyo, 1956, p. 1.
8) M. Hyduck und H. Haehn, *Biochem., Z.*, **128**, 568 (1922).

* The fermenting activity was determined at intervals of 10 min or 20 min with a shaking apparatus. Q_{CO_2} (max): the value calculated from the largest volume of CO_2 produced in 20 min. Q_{CO_2} (20′–40′, 120′–140′, etc.): the value calculated from the volume of CO_2 produced in 20 min and suffixed with the time elapsed from the start of measurement. t′; time (in minutes) required for observing Q_{CO_2} (max)

were used in order to investigate the effect of various drying methods and it was found that this effect differs in each value. It is therefore necessary to determine whether all of these values are indispensable, since it is inconvenient to use so many values in comparing the fermenting power between many samples. Then arises the following questions. How many values are necessary to express the fermenting power of dry bakers' yeast? What values should be taken for it? What is the physiological significance of those values? Clarification of these questions will lead to the elucidation of the mechanism of fermentation of dry bakers' yeast. In this report the former two questions are investigated.

EXPERIMENTAL AND RESULTS

All samples used in this experiment were dry bakers' yeasts prepared by methods reported previously[9][10]. The material for preparing the dry yeast was entirely taken from commercial bakers' yeast. An adequate amount of fresh yeast was pushed out on filter papers through a 20-mesh sieve and dried at 30°~35°C with a Faust-Heim aeration drier for from three to five hours, while some samples were dried by several effective methods cited in the author's previous reports[9], e.g., supply of ethanol vapour, addition of glucose, and drying in vacuum. Dry yeast prepared by the above methods was used not only soon after drying but also after being stored in a sample tube tightly closed at 0° or 30°C. Thus, this experiment was carried out using as many samples as possible in order to investigate a most suitable universal method for determination of fermenting power of dry bakers' yeast.

Determination of the fermenting activity was made as follows according to the method hitherto employed[10]. Dry yeast 0.7 g, was weighed into a vessel; 10 ml of the medium composed of 7% sucrose, 0.25% potassium dihydrogen phosphate, 0.25% ammonium dihydrogen phosphate, previously warmed at 30°C, was poured into the same vessel, and the dry yeast was sufficiently suspended by occasional shaking for ten minutes. In the next ten minutes, the air of this vessel was exchanged with carbon dioxide and the vessel put into the shaking apparatus, kept at 30°C in a water tank, and shaken till the start of determination at which time the escape valve was closed, so as to warm both the content and the vessel to 30°C. Then, while the vessel was being shaken at 30°C, the evolved carbon dioxide was caught in a gas-burette, and its volume was measured at ten or twenty-minute intervals.

Fig. 1 shows the total volume of carbon dioxide evolved from the start in each measurement and the volume evolved during twenty minutes between two successive measurements. The latter volume is represented as the curve of fermenting activity at each time,

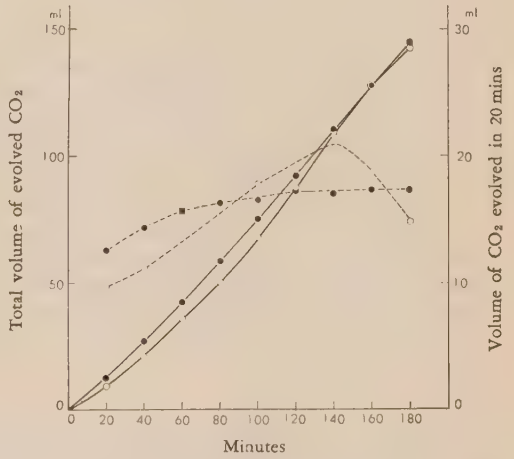

FIG. 1. Fermentation of Dry Yeast

○ sample A; ● sample B; —— total volume
- - - - volume in 20 mins

and this curve is generally divided into three parts: periods of increasing, maximum, and decreasing activities. So the "induction period" (abbreviated as I.P.) was defined as follows:

$$\text{I.P.} = t' + 20' - (20' \times \text{increasing } CO_2 / \text{max } CO_2)$$

In the above equation, max CO_2 expresses the volume of carbon dioxide evolved during twenty minutes of the maximum activity, i.e., the largest volume of the gas produced in twenty minutes. The increasing CO_2 represents the total volume in the period of increasing activity, and t' represents time (in minutes) elapsed till the beginning of the production of the max CO_2, i.e., the time required for observing Qco_2(max) as stated above. The figure 20' in t'+20' means the time required from the preparation of suspensions of the dry yeast till the start of measurement, and the volume of carbon dioxide evolved during this time was estimated

9) M. Takakuwa, *J. Fermentation Technol.* (Japan), **33**, 104 (1955); *ibid.*, 141 (1955).
10) M. Takakuwa, *Sci. Rep. Matsuyama Agric. Coll.*, **12**, 39 (1954).

from the volume of gas that was evolved since the beginning of measurement. The increasing CO_2 is also included in this volume. Such a definition for "induction period" is a little different from that of Katagiri but is very similar to Sobotka's definition.

(1) Expression of Dry Yeast Fermenting Power

Fig. 1 shows that fermenting activity changes with the progress of fermentation and that this change is more distinct in the curve of fermenting activity than in the curve of the total volume of carbon dioxide evolved. However, for the purpose of comparing the fermenting power between many samples, it is more convenient to have this curve represented by a few values. H. Sobotka[11] used "Halbgärzeit" which had been defined by Willstätter et al.[1] after examining various methods for determining fermenting activity. Oyaas[12] also measured fermenting activity with a shaking apparatus, but he applied the total volume of carbon dioxide produced in one hundred and fifty minutes from the start of measurement. Shinma et al.[13] measured the evolved carbon dioxide at fixed intervals in a test of Meissl's method, and reported that the uniform state of fermentation appears between the one hundredth and three hundredth minute, and represented a fermenting activity with the value obtained dividing the mg of the evolved carbon dioxide by the number of hours required for its production.

However, only one value is shown in each of these experiments, and the meaning of such a value, namely, the relation between this value and the above curve, has not yet been studied in details. When Sobotka compared many fermenting powers adopting the value of "Halbgärzeit", he concluded that fermenting activity would become "uniform" in "Halbgärzeit", and calculated I.P. in accordance with this conclusion.

But it could not be found whether fermenting activity becomes uniform when one half of sucrose has been fermented. In this experiment, which was conducted in almost the same condition as that of Sobotka's, the percentage of the total volume of carbon dioxide produced before the beginning of the production of max CO_2, i.e., the "uniform" state of fermentation was examined. As shown in Fig. 2, Sobotka's conclusion was rejected at a 5% level of significance. Therefore, it is impossible to conclude that fermenting activity will be uniform in "Halbgärzeit".

The value of I.P. is important as one of the characteristic properties of dry yeast. Correlation coefficients and ratios between I.P. and max CO_2 or t′ are shown in Table I. A negative correlation was found between max CO_2 and I.P., and a very high positive correlation ($r=0.956$) between I.P. and t′. Considering the degree of correlation between max CO_2 and I.P., it was concluded that the fermenting power of dry yeast cannot be completely represented by only one value.

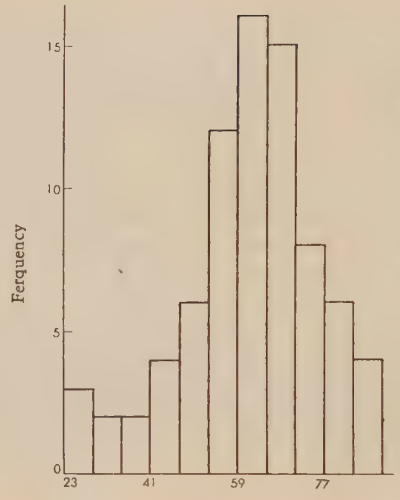

FIG. 2. Distribution of the Number of Samples in the Ratio of CO_2 evolved before reaching max CO_2 to the Total.

A shows the ratio of the volume of CO_2 evolved before reaching max CO_2 to the total of CO_2 with %. 77 samples were used.

Therefore, the curve of fermenting activity as shown in Fig. 1 was analysed with orthogonal polynomial. When 35 samples were taken at random, most of them could be expressed in quadratic equations, and it was confirmed through Bayes' theorem that the curve will be expressed by the polynomial whose degree is less than three. In other words, when fermenting activity is shown by Y, Y can be expressed in a quadratic form of t (the time of fermentation). Thence it will be most reasonable to express the fermenting power of dry yeast by such three values that can decide a, b, c in a quadratic equation: $Y=at^2+bt+c$.

(2) Correlation of $Q_{CO_2}(20'-40')$ to $Q_{CO_2}(max)$ and ΔQ_{CO_2}

ΔQ_{CO_2} represents the increase of Q_{CO_2} in an hour

11) H. Sobotka, *Z. für physiol. Chem.*, **134**, 1 (1924).
12) J. Oyaas, M. J. Johnson, *Ind. Eng. Chem.*, **40**, 280 (1948)
13) K. Shinma, T. Kada, and M. Ikeda, unpublished.

TABLE I. CORRELATION OF INDUCTION PERIOD TO max CO_2 OR t'

		Region[a]		Correlation			
		max	min	coefficient[b]	ratio[c]	F	Fo[d]
To max CO_2	I.P.	181	0	$r=-0.644$	$\eta_{yx}=0.714$	1.53	2.07
	max CO_2	30.5	12.4	$-0.491>\rho>-0.759$	$\eta_{xy}=0.761$	1.33	1.82
To t'	I.P.	181	0	$r=0.956$	$\eta_{yx}=0.975$	2.68	1.81
	t'	350	10	$0.972>\rho>0.932$	$\eta_{xy}=0.972$	1.95	1.82

77 samples were used.

a: Region in which values were measured. max CO_2 is represented in ml units.

b: Confidence limit of ρ (population) was calculated at the 5% level of significance.

c: I.P. is expressed as x.

d: Fo shows the value of Fo which makes Pr {F>Fo} equal to 5% in F-distribution. F is a calculated value. In the case of F>Fo, the hypothesis of linearity is to be rejected at levels less than the 5% level of significance.

TABLE II. CORRELATION OF $Q_{CO_2}(20'-40')$ TO $Q_{CO_2}(max)$ OR INCREASE OF Q_{CO_2}

	N[a]		Region		Correlation			
			min	max	coefficient	ratio	F	Fo
To $Q_{CO_2}(max)$	226	Q_{CO_2} (20'–40')	13	307	$r=0.904$	$\eta_{yx}=0.930$	2.50	1.55
		Q_{CO_2} (max)	73	307	$0.926>\rho>0.876$	$\eta_{xy}=0.929$	2.93	1.60
To increase of Q_{CO_2}	628	Q_{CO_2} (20'–40')	2	119	$r=0.693$	$\eta_{yx}=0.745$	10.1	1.85
		ΔQ_{CO_2}	−13	41	$0.731>\rho>0.651$	$\eta_{xy}=0.739$	7.32	1.78
To $Q_{CO_2}(max)$	299	Q_{CO_2} (20'–40')	121	226	$r=0.962$	$\eta_{yx}=0.965$	4.29	2.25
		Q_{CO_2} (max)	121	229	$0.969>\rho>0.953$	$\eta_{xy}=0.964$	1.64	2.25

a: N indicates the number of samples used.

which is calculated from the difference between $Q_{CO_2}(20'-40')$ and $Q_{CO_2}(60'-80')$. If $Q_{CO_2}(20'-40')$ is regarded as initial fermenting activity, a, b, and c in the above quadratic equation can be decided with three measuaed values: $Q_{CO_2}(20'-40')$, ΔQ_{CO_2}, $Q_{CO_2}(max)$. It was confirmed that a high correlation coefficient exists between $Q_{CO_2}(20'-40')$ and $Q_{CO_2}(max)$ (Table II). However, considering the standard deviations in Fig. 3, it might be deduced that a higher correlation coefficients will exist when $Q_{CO_2}(20'-40')$ is larger than approx. 110. In other words, when $Q_{CO_2}(20'-40')$ is smaller than approx. 110, variance of the expected $Q_{CO_2}(max)$ is apt to become larger. Now, the number of samples which indicate $Q_{CO_2}(max)$ before the eightieth minute from the start, decreases sharply in the region of 110 and 120 of $Q_{CO_2}(20'-40')$, as it is shown with the ratio to the total number of samples in each class in Fig. 4.

With samples of which $Q_{CO_2}(20'-40')$ exceeded 120, it was confirmed that an almost perfect positive correlation exists between $Q_{CO_2}(20'-40')$ and $Q_{CO_2}(max)$, and it was noticed that most of the samples having especially large $Q_{CO_2}(20-40')$ values have already shown $Q_{CO_2}(max)$ at the twentieth minute after the start.

(3) Correlation between $Q_{CO_2}(20'-40')$ and the Value of Dough Fermentation Test

As for the fresh yeast, the correlation between the fermenting power and baking power was examined by Sato et al.[14], while for dry yeasts, this correlation has not yet been examined. There are diverse views on methods of testing baking power[15),16)], but in this experiment, the dough fermentation test[4)] was carried out and the volumes of dough at the first period of fermentation (one hundred and five minutes) and that at the second one (fifty minutes after the first one) were measured. It was confirmed that a high positive correlation exists between $Q_{CO_2}(20'-40')$ and values of the dough fermentation test (Table III). These values were much higher than those reported in Sato's paper[6)] (for

14) T. Sato and H. Hioki, *Rept. Food Research Inst.* (Tokyo), **No. 6**, 47 (1952).

15) R. Gullemet, F. Duffar, and A. Bourdet, *Congr. intern. inds. fermentation, Confs. et communs.*, **1947**, 344.

16) M. Chikasue and H. Matsumoto, *J. Fermentation Technol.* (Japan), **27**, 79 (1949); *ibid.*, 194 (1949); H. Matsumoto, *ibid.*, **30**, No. 10, 263 (1952).

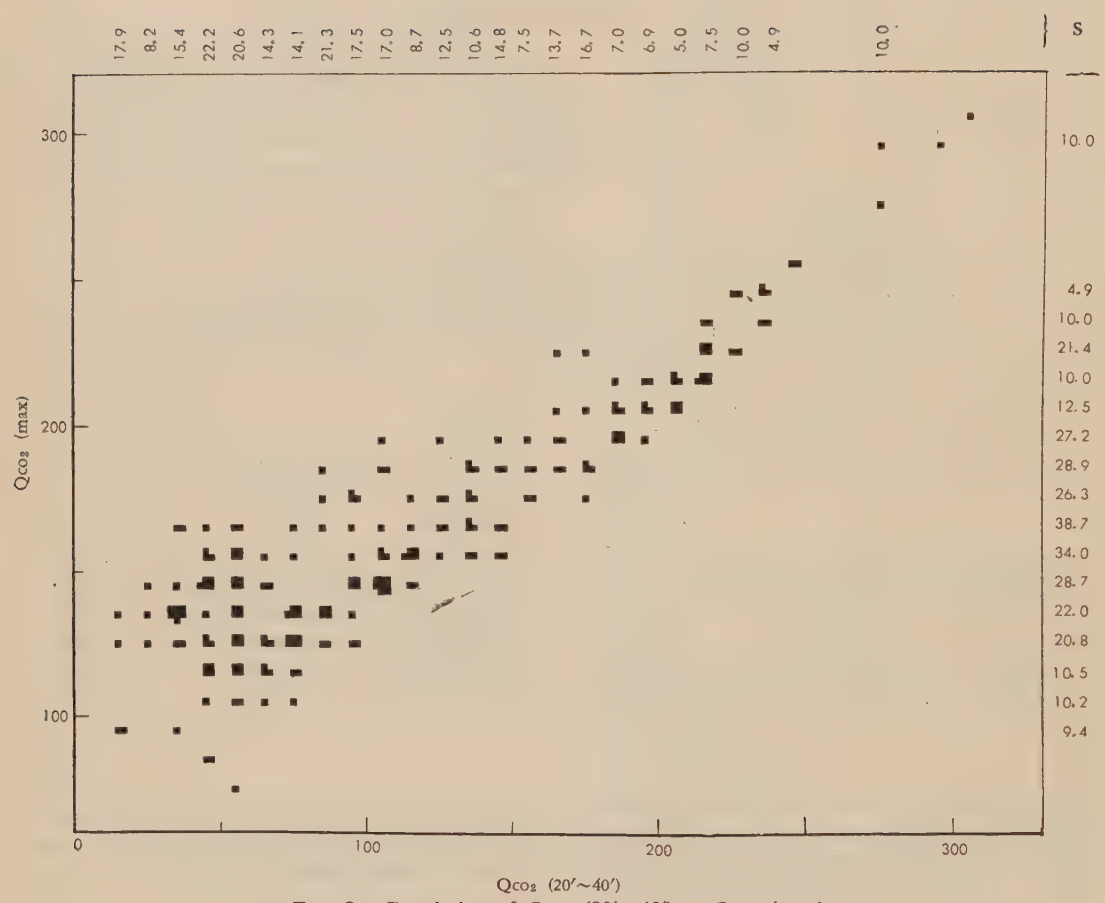

FIG. 3. Correlation of Qco_2 (20′~40′) to Qco_2 (max)
S shows the standard deviation of Qco_2 (20′~40′) or Qco_2 (max) in each class.

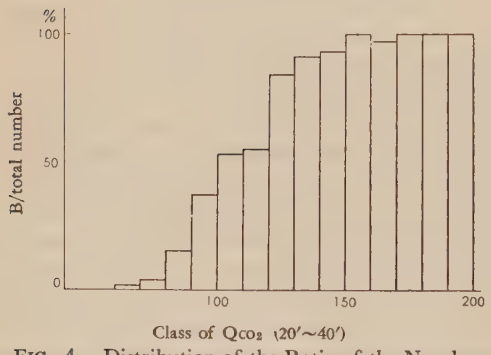

FIG. 4. Distribution of the Ratio of the Number of Samples, which had reached Qco_2(max), to the Total.
B shows the number of samples which had reached Qco_2(max).

the volume of dough at the first period: r=0.282, for the volume at the second period: r=0.384).

DISCUSSION

Qco_2(20′–40′) may be used not only as a criterion for estimating fermenting power but also in case of baking power, because a high positive correlation exists between the Qco_2(20′–40′) and value of the dough fermentation test as well as between that and Qco_2(max) or ΔQco_2.

But, considering the fact that the curve of fermenting activity can be expressed as a quadratic function of t, it is reasonable to ex-

TABLE III. CORRELATION of $Qco_2(20'-40')$ TO TEST OF DOUGH FERMENTATION

		Region		Correlation			
		min	max	coefficient	ratio	F	Fo
To D.F. (1st)[a]	Qco_2 (20′–40′)	35	211	$r=0.794$	$\eta_{yx}=0.899$	2.77	2.36
	D.F. (1st)	210	470	$0.893>\rho>0.623$	$\eta_{xy}=0.870$	1.16	2.54
To D.F. (2nd)	Qco_2 (20′–40′)	35	211	$r=0.734$	$\eta_{yx}=0.894$	3.84	2.36
	D.F. (2nd)	210	430	$0.860>\rho>0.527$	$\eta_{xy}=0.819$	1.13	2.76

34 samples were used.

a: D.F. (1st) shows the 1st period in the test of dough fermentation and D.F. (2nd) shows the 2nd.

amine ΔQco_2 and other values in addition to $Qco_2(20'–40')$ for the purpose of comparing various kinds of dry yeasts, because the influence of factors other than $Qco_2(20'–40')$ becomes larger when $Qco_2(20'–40')$ is smaller than 120. Though fresh intact yeasts show constant fermenting activity from the start of measurement[1], when they are dried and stored it is revealed that their fermenting activity varies. Nilsson et al. report on the breaking of organic interrelation of various enzyme systems within the dried cell[17], and the present author has also obtained the same result[18]. So, it may be considered that ΔQco_2 and $Qco_2(max)$ are related to the retention of other enzyme systems than zymase in the cell. It will surely be beneficial to study the significance of these values in regard to cell physiology in clarification of the change of cells during dehydration and storage.

Besides, the value of the dough fermentation test had a large variance in samples having large $Qco_2(20'–40')$ values in which an almost perfect positive correlation existed between $Qco_2(20'–40')$ and $Qco_2(max)$. This may be ascribed to the influence of some factors[19] other than the above-mentioned three.

SUMMARY

The volume of carbon dioxide evolved in a shaking fermentation apparatus was measured at fixed intervals in the same way as stated in the previous paper. Then, the curve showing the rise and fall of fermenting activity was analysed with orthogonal polynomial. This method for the determination of fermenting activity was compared with usual methods of expressing fermenting power, from which the author's method was proved to be more reasonable than the others, particularly, in point of deciding initial fermenting activity.

1) It was confirmed that the fermenting power of dry yeast can be expressed in a polynomial lower than cubic, and then that this can be further represented by three factors: an initial fermenting activity (measured as $Qco_2(20'–40')$), a power which increases or recovers fermenting activity (ΔQco_2), a miximum fermenting activity ($Qco_2(max)$).

2) The correlation between the above values, I. P., and t' was examined respectively. Thereupon, the following connections were confirmed: there exists a negative correlation between $Qco_2(max)$ and I. P., an almost perfect positive correlation between I. P. and t' and a positive correlation between $Qco_2(20'–40')$ and ΔQco_2 as well as between $Qco_2(20'–40')$ and $Qco_2(max)$.

3) In the case of a small $Qco_2(20'–40')$ value, the influence of ΔQco_2 and the other factors seems to be larger, because the expected $Qco_2(max)$ has a large variance, but generally $Qco_2(20'–40')$ may be used as a criterion to indicate fermenting power of dry yeast.

4) Also, a positive correlation exists between $Qco_2(20'–40')$ and the value of the dough fermentation test, but this is smaller than the value observed between $Qco_2(20'–40')$ and

17) R. Nilsson und F. Alm, *Biochem. Z.*, **286**, 254 (1936).

18) M. Takakuwa, unpublished.

19) L. Atkin, A. S. Schultz, and C. N. Frey, "Enzymes and their ole in wheat technology", Interscience publishers, New York, N. Y., 1946, p. 321.

$Q_{CO_2}(max)$. Therefore it is concluded that the influence of factors other than the above three may be stronger in dough fermentation than in sucrose fermentation.

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Microbiological Studies of *Coli-aerogenes* Bacteria*

Part VI. The Action of Antibiotics on Bacterial Respiration and α-Ketoglutaric Acid-Fermentation

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Dihydrostreptomycin not only depressed the bacterial accumulation of α-ketoglutarate from a variety of carbonaceous substances including glucose, acetate, pyruvate, lactate and C_4-dicarboxylic acids such as succinate, fumarate and malate, but also stimulated the bacterial oxidation of α-ketoglutarate under suitable conditions. The dihydrostreptomycin-resistive strains were found to induce α-ketoglutaric acid-fermentation, and an explanation was put forward for the mechanism of development of drug-resistance. Chloramphenicol was observed to inhibit the α-ketoglutarate-synthesizing system in the bacteria without causing any inhibition of the oxidative removal of a variety of carbonaceous substances. It was concluded that the bacteria possessed both oxidation or respiration system ; one was sensitive to the drug and the other non-sensitive. The mode of action of streptomycin or chloramphenicol upon the formation of an adaptive enzyme (protein synthesis) was discussed here, based on the results of present investigations. Tetracycline and the related compounds were ascertained to greatly inhibit the consumption of substrates by the bacteria.

INTRODUCTION

A number of the studies carried out on the mode of action of various antibiotics have demonstrated that a given metabolic process or enzyme reaction is inhibited by the drug. On the other hand, it has been reported that the lethal effects of antibiotics on microorganisms greatly depend upon the environmental conditions to which the organisms are exposed[1~4].

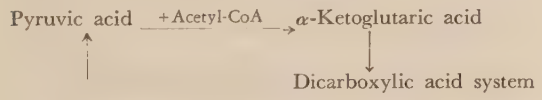
* A part of this paper was read before the Annual Meeting of the Japanese Biochemical Society (July, 1957).

1) T.F. Paine, Jr, and L. S. Clark, *Antibiotics and Chemotherapy*, **4**, 262 (1954).
2) E. D. Rosenblum and V. Bryson, *Antibiotics and Chemotherapy*, **3**, 957 (1953).
3) A. E. Wasserman, *Antibiotics and Chemotherapy*, **3**, 977 (1953).
4) C Hurwitz and C. L. Rosano, *J. Bacteriol.*, **75**, 11 (1958).

Among the various metabolic activities which have been reported to be greatly affected by the antibiotics, the authors' attention has been directed to the metabolism of carbohydrates and organic acids related to the terminal respiration of the bacteria of *coli-aerogenes*.

In the previous papers[5~8], the authors have studied the oxidative fermentations of carbohydrates and organic acids which belong to the terminal respiration system, by the bacteria of *coli-aerogenes* type, and have demonstrated that these bacteria produce α-ketoglutaric acid as the principal fermentation product under aerobic conditions, that is to say, these bacteria induce α-ketoglutaric acid-fermentation. It has been concluded from further investigations on the oxidative fermentation by the bacteria that the major production of α-ketoglutaric acid from glucose, pyruvate lactate, acetate and C_4-dicarboxylic acids does not proceed by way of the conventional tricarboxylic acid cycle, but occurs by way of a certain reaction which has been termed by the authors[8] as pyruvate-acetate reaction since not only the bacterial strains of *E. coli* but also those of *A. aerogenes* have been found to reveal a very poor ability of producing α-ketoglutaric acid from tricarboxylic acids, whereas, they have a very high activity of producing isocitritase by which both glyoxylate and succinate are formed from isocitrate[9].

It has consequently been propounded that a new metabolic cycle (the α-ketoglutaric acid cycle) might occur, in which the following pathway would be possible[8,9]:



The present investigation has been undertaken in order to elucidate what influence would be exerted by the presence of various antibiotics on oxidative fermentation and respiration in the bacteria of *coli-aerogenes*. Antibiotics which were employed in the present investigation were dihydrostreptomycin-sulfate, chloramphenicol and tetracycline itself and the related compounds.

5) H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 210 (1957).
6) H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 215 (1957).
7) H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 346 (1957).
8) H. Katagiri and T. Tochikura, This Bulletin, **21**, 351 (1957).
9) H. Katagiri and T. Tochikura, This Bulletin, **22**, 143 (1958).

EXPERIMENTAL AND RESULTS

The experimental procedure was performed by methods mentioned in the previous papers, except that succinate and fumarate were determined by the methods of Krebs[10] and Marshall et al.[11], respectively.

Action of Dihydrostreptomycin.

The most fundamental mode of the antibiotical action of streptomycin has yet not been elucidated, although various studies have been performed with both proliferating and nonproliferating cells of sensitive and resistive strains of bacteria. At present, the following reactions are well known to be greatly affected by streptomycin or dihydrostreptomycin: (1) formation of adaptive enzyme[12~14,50~51], (2) metabolism of nucleic acid[15], (3) oxidation of amino acid[18~20], (4) respiration system[20~28], (5) diamine oxidase activity[16~17].

Effective studies on the mode of action of streptomycin have been performed by Geiger and subsequently Umbreit, Oginsky and Smith. Geiger observed the inhibition of streptomycin on the oxidation of aspartate by *E. coli* and it was demonstrated by him that if fumarate was oxidized by the organism prior to the addition of amino acids such as aspartate and serine, a striking increase would occur in oxidation of the amino-acids, whereas, if streptomycin was present in the course of the oxidation of fumarate, such increase would not be observed[18]. Umbreit has recognized significance of the investigation carried out by Geiger and has demonstrated that the terminal respiration system in *E.*

10) H. A. Krebs, *Biochem. J.*, **31**, 2095 (1937).
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12) R. J. Fitzgerald and F. Bernheim, *J. Bacteriol*, **55**, 765 (1948).
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14) Y. Yamamura and T. Sasakawa, *Symposia on Enzyme Chemistry* **5**, 60 (1950).
15) S. S. Cohen, *J. Biol. Chem.*, **166**, 393 (1946).
16) C. A. Owen., Jr., A. G. Karlson and E. A. Zeller, *J. Bacteriol.*, **62**, 53 (1951).
17) E. A. Zeller., C. A. Owen, Jr. and A. G. Karlson, *J. Biol. Chem.*, **188**, 623 (1951).
18) W. B. Geiger, *Arch. Biochem.*, **15**, 227 (1947).
19) K. Wight and D. Burk, *Antibiotics and Chemotherapy*, **1**, 379 (1951).

Table I. Influence of Dihydrostreptomycin upon Production of α-Ketoglutaric Acid by Washed Cells of *E. Coli* from Glucose, Pyruvic, Lactic, and Acetic Acids

Exp. No.	I		II		III		IV		V	
Substrates (mM)	Glucose*		Pyruvate		Pyruvate plus acetate		*l*-Lactate		Acetate	
	4.000		4.000		3.000	2.000	3.300		4.000	
Dihydrostreptomycin added (mg/40 ml)	0	20	0	30	0	20	0	40	0	40
Washed cells of *E. coli* (mg)	300	300	250	250	130	130	130	130	340	340
Time of incubation (hours)	15	15	13	13	12	12	13	13	15	15
					Pyruvate consumed					
Substrates consumed (mM)	4.000	4.000	4.000	4.000	3.000	3.000	3.300	3.300	4.000	4.000
α-Ketoglutaric acid found (mM)	1.630	0	0.420	0.123	0.952	0.132	0.412	0.141	0.127	0

* $CaCO_3$ (3 mM) was added.

Table II. Action of Dihydrostreptomycin on Production of α-Ketoglutaric Acid by Washed Cells of *E. Coli* from C_4-Dicarboxylic and Acetic Acids

Substrates	Fumarate plus acetate		Succinate		Succinate plus acetate		DL-Malate plus acetate	
(mM)	2.000	1.500	2.000		2.000	2.000	2.000	1.500
Dihydrostreptomycin added (mg/50 ml)	0	20	0	20	0	20	0	20
Dicarboxylic acids consumed (mM)	2.000	2.000	2.000	2.000	2.000	2.000		
α-Ketoglutaric acid found (mM)	0.425	0.258	0.300	0.100	0.600	0.210	0.414	0.200

coli grown anaerobically, is inhibited by the presence of streptomycin[20].

It has been concluded by Umbreit et al.[20,23~24] and Oginsky et al.[21] that the terminal respiration system in *E. coli* grown anaerobically, involves a pyruvate-oxalacetate condensation and streptomycin exerts the inhibiting effects on this condensation reaction. Furthermore, they have shown that the process of terminal respiration inhibited by streptomycin is of a different type from the conventional tricarboxylic acid cycle, since both the formation of citrate from oxalacetate and pyruvate, and the oxidation of citrate by *E. coli* are found to be not inhibited by the presence of streptomycin[20~25].

The studies mentioned above have led the authors to learn whether or not there exists relationship between streptomycin and α-ketoglutaric acid-fermentation by *coli-aerogenes*. Experiments were instituted with *E. coli* (G-2 strain) in the following manner. After being grown with a shaker at 30°C for 2 days on a medium containing 2% glucose, 0.1% $(NH_4)_2HPO_4$, 0.3% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% NaCl, 5 mg% $FeSO_4 \cdot 7H_2O$ and 1% $CaCO_3$, *E. coli* was collected by a centrifuge and washed with distilled water. To the washed cells (130~340 mg as dried matter) were added (1) 5 mM phosphate buffer (pH : 6.5) (2) 50 μM $MgSO_4$ (3) various substrates (Na-salts in the case of organic acids) (4) 0~40 mgdi hydrostreptomycin and water to make the total volume up to 40 ml. The reaction mixture was placed on a shaker and incubated for 12~15 hours at 30°C.

From the results of experiments given in Table I, it has been found with the washed cells of *E. coli* that the production of α-ketoglutarate from a variety of substrates is strongly inhibited by the presence of dihydrostreptomycin, although the consumption of substrates are not greatly affected at the end period of

20) W. W. Umbreit, *J. Biol. Chem.*, **177**, 703 (1949).
21) E. L. Oginsky, P. H. Smith and W. W. Umbreit, *J. Bacteriol.*, **58**, 747 (1949).
22) P. H. Smith, E. L. Oginsky and W. W. Umbreit, *J. Bacteriol.*, **58**, 761 (1949).
23) W. W. Umbreit, P. H. Smith and E. L. Oginsky, *J. Bacteriol.*, **61**, 595 (1951).
24) W. W. Umbreit and P. Heneage, *Bacteriol. revs.*, **17**, 39 (1953).
25) W. W. Umbreit, *J. Bacteriol.*, **66**, 74 (1953).

Table III. Influence of Dihydrostreptomycin upon Oxidation of a Variety of Substrates by Washed Cells of *E. Coli*

Substrates (μM)	Bacterial cells (mg)	Time of incubation (min.)	Dihydrostreptomycin added (γ/2.3 ml)	O_2 uptake μl
Acetate 10	7	180	0	371
			3000	370
Pyruvate 10	7	180	3	410
			3000	415
Succinate 30 plus acetate 60	8	120	0	907
			1000	985
DL-Malate 30	8	120	0	807
			1000	898
Fumarate 30 plus acetate 60	8	120	0	728
			1000	810

Preincubation for 60~120 min at 37°C with or without dihydrostreptomycin in absence of substrates

Table IV. Influence of Dihydrostreptomycin upon Oxidation of α-Ketoglutarate by Washed Cells of *E. Coli*

Exp. No.	I——a	I——b	II——a	II——b
Addition of dihydrostreptomycin (mg/50 ml)	0	40	0	50
Addition of α-ketoglutarate (mM)	1.280	1.280	1.000	1.000
Washed cells of *E. coli* (mg)	280	280	100	100
Time of incubation (hours)	20	20	12	12
α-Ketoglutarate consumed (mM)	0.504	1.280	0.177	0.774

incubation. These results were again ascertained with C_4-dicarboxylic acids such as succinate, fumarate and malate, singly or together with acetate as the substrate. The results of experiments with dicarboxylic and acetic acids are given in Table II. Incubations were carried out on the shaker at 30°C for 12 hours in the presence or absence of dihydrostreptomycin on the reaction mixture (50 ml) containing 5 mM phosphate buffer (pH; 5.3), 50 μM $MgSO_4$, 130 mg washed cells of *E. coli* and stated amounts of Na-salts of dicarboxylic and acetic acids. As it is seen in Table II, even under the conditions in which dihydrostreptomycin is present at a level of 400 γ per ml, the oxidations of dicarboxylic acids by *E. coli* would occur but the accumulation of α-ketoglutaric acid is remarkably decreased.

The action of dihydrostreptomycin upon the terminal respiration in *E. coli* was also investigated by the use of a conventional Warburg technique; the reaction mixture contained 200 μM phosphate buffer (pH: 6.0~6.7), 4 μM $MgSO_4$, various amounts of substrates (Na-salts), 7~8 mg (as dry weight) washed cells of *E. coli* grown aerobically on glucose medium, and in the centre well, 0.2 ml 15% KOH; total volume 2.5 ml. Incubations were carried out in either the presence or absence of dihydrostreptomycin at 37°C in air.

As will be seen in Table III, there was no inhibiting action of dihydrostreptomycin upon the oxidation of substrates to be observed. It has already been found by Umbreit and coworkers that the oxidation of citric acid as well as "acetate-oxalacetate condensation reaction" are not inhibited by the presence of streptomycin[23,24].

Stimulating Effect of Dihydrostreptomycin on Oxidation of α-Ketoglutaric Acid

In the course of investigation on the mode of action of streptomycin on the formation of α-ketoglutaric acid from varied carbonaceus substances, the authors have found that the bacterial oxidation of externally added α-ketoglutaric acid is greatly stimulated by this antibiotic under suitable conditions.

Experiments were carried out with washed cells of *E. coli* (G–2 strain) grown aerobically on a glucose medium in the same manner as previously mentioned. The results are shown in Table IV, in which incubation was carried out for 12~20 hours at 30°C on the shaker

26) T. Sasakawa, *Symposia on Enzyme Chemstry*, **8**, 98 (1953).
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TABLE V. INFLUENCE OF ADDITION OF SUCCINATE AND GLUCOSE UPON OXIDATION OF α-KETOGLUTARATE BY WASHED CELLS OF *E. COLI* IN PRESENCE AND ABSENCE OF DIHYDROSTREPTOMYCIN

Exp. No.	I——a	I——b	II——a	II——b	II——c
Additions					
Dihydrostreptomycin (mg/50 ml)	0	40	0	15	37
α-Ketoglutarate (mM)	1.280	1.280	1.000	1.000	1.000
Glucose (mM)	0	0	0.556	0.556	0.556
Succinate (mM)	2.000	2.000	0	0	0
Washed cells of *E. coli* hours' (mg)	300	300	100	100	100
Analysis after 15 hours' incubation					
Glucose consumed (mM)			0.556	0.556	0.556
α-Ketoglutarate found (mM)	1.685	0.357	0.843	0.100	0.100
Pyruvate and acetate found	0	0	0	0	0

Each vessel contained 5 mM phosphate buffer (pH: 6.5), 50 μM $MgSO_4$ and the stated amounts of additions; total volume 50 ml. Incubation for 15 hours at 30°C on a shaker.

TABLE VI. INFLUENCE OF ADDITION OF GLUCOSE AND AMMONIUM CHLORIDE UPON OXIDATION OF α-KETOGLUTARATE BY WASHED CELLS OF *E. COLI* IN PRESENCE AND ABSENCE OF DIHYDROSTREPTOMYCIN

Exp. No.	I	II	III	IV	V	VI	VII	VIII
Additions								
Dihydrostreptomycin (mg/50 ml)	0	0	21	0	21	0	0	21
α-Ketoglutarate	0	1.000	1.000	1.000	1.000	0	1.000	1.000
Glucose (mM)	0.556	0	0	0.556	0.556	0.556	0.556	0.556
NH_4Cl (mM)	0	0	0	0	0	0.93	0.93	0.93
Analysis after incubation								
Consumption of glucose (%)	100.0			100.0	100.0	100.0	100.0	100.0
Consumption of α-ketoglutarate (%)		30.6	35.3	10.7	40.0		30.0	83.3

Each vessel contained 5 mM phosphate buffer (pH: 6.6), 200 mg washed cells of *E. coli*, 50μ M $MgSO_4$ the stated amounts of additions and water to 50 ml; incubation for 7 hours at 30°C on a shaker

in the presence or absence of the drug with the media containing 5 mM phosphate buffer (pH: 6.5), 50 μM $MgSO_4$, the stated amounts of Na-α-ketoglutarate and washed cells of *E. coli*, and water to make the total volume up to 50 ml.

However, there was no noticeable effect of dihydrostreptomycin on the bacterial oxidation of α-ketoglutarate to be observed, when the incubation lasted for a short period. Further investigations now reveal that dihydrostreptomycin tends to stimulate the bacterial oxidation of α-ketoglutarate under the conditions in which available nitrogen and carbon substances are present.

The experimental results are shown in Table V and VI. Table V shows that dihydrostreptomycin exerts a much more stimulating effect on the bacterial oxidation of α-ketoglutarate when an available carbon source such as glucose and succinate is present than when it is absent. It will, however, be shown in Table VI that the addition of both available carbonaceus substances and nitrogen sources bring about most increase in the bacterial oxidation of α-ketoglutarate in the presence of dihydrostreptomycin.

Oxidative Fermentation in Dihydrostreptmycin Resistive Strain of *E. coli*

The results hitherto mentioned may provide an experimental approach to some problems concerning the development of bacterial resistance to antibiotics. The authors investigated the oxidative fermentation in dihydrostreptomycin-resistive strains of *E. coli*. *E. coli* (G-2 strain) was rendered dihydrostreptomycin-resistant by repeated subculturing in increasing amounts of dihydrostreptomycin on the broth containing 1% each of chrysalis extract and peptone and 0.5% NaCl.

According to this procedure, resistive strains were obtained which were able to grow even in the presence of 30 mg dihydrostreptomycin per ml of broth.

Table VII. Oxidative Fermentation of Glucose by Dihydrostreptomycin-Sensitive and Resistive Strains of *E. Coli*

	Sensitive strain (parent)	14000γ/ml resistant		28000γ/ml resistant	
Addition of dihydrostreptomycin (γ/ml)	0	0	14000	0	14000
Consumption of glucose (%)	92	98	100	95	100
α-Ketoglutarate produced (% on consumed glucose)	29.2	28.9	31.1	29.2	31.0
Pyruvate produced	0	0	0	0	0

Oxidative fermentations were carried out with the resistive strains in the following manner. In this case, an experiment with the sensitive strain was performed for the sake of comparison. Both strains were grown in the presence or absence of dihydrostreptomycin on a medium containing 2.0% glucose, 0.05% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.04% $MgSO_4 \cdot 7H_2O$ and 0.2% NaCl to which sterilized $CaCO_3$ (2 g per 100 ml) was added at the time of inoculation. Thirty ml of the medium was taken in a 500-ml flask and sterilized. After inoculation with 1 ml of 48 hours' cultures of both strains on bouillon, the flask were placed on the shaker. After incubation for 4 days at 30°C, the fermentation products were analyzed according to the methods described in the previous papers.

From the results shown in Table VII, it has now been found that under aerobic conditions, dihydrostreptomycin-resistive strains not only reveal high activity of metabolizing glucose during their growth, but they also produce α-ketoglutaric acid in a very large quantity.

In case of sensitive strains, α-ketoglutaric acid-fermentation occurred in the absence of dihydrostreptomycin, but growth was strongly inhibited in its presence.

Based on intensive and extensive studies of the metabolism of the resistant mutant, it has already been indicated that drug-resistance is accompanied with the elimination of specific metabolic process or of those enzymes to which the drug acts as an inhibitor, and also accompanied with development of the alternative metabolic process no longer inhibited by the drug[22)29~33)].

Umbreit and his associates[22)] have demonstrated that resistant and dependent variants which are derived from the streptomycin-sensitive strain of *E. coli*, no longer possess a pyruvate-oxalacetate condensation by which pyruvate is oxidized in the course of respiration, and that this condensation itself is indeed the reaction inhibited in streptomycin-sensitive strains of *E. coli*.

Thus, they have considered that the ability to grow in the presence of streptomycin depends upon the development of unknown reactions which permit the bacterial cells to dispense with this pyruvate-oxalacetate reaction.

On the other hands, the present investigations have revealed the fact that there is no difference to be observed, between the oxidative metabolism of the dihydrostreptomycin-sensitive strain and that of the resistive strain of *E. coli*, since the resistive variants are observed to be capable of metabolizing glucose aerobically with an extreme readiness and also of inducing α-ketoglutaric acid-fermentation as in the case of the sensitive parent in the absence of dihydrostreptomycin. It should, however, be noted that the metabolic process of α-ketoglutaric acid in the dihydrostreptomycin-sensitive parent is greatly affected by the presence of this antibiotic as already mentioned.

From these observations, the authors have presented an explanation that antibiotic-resistance is not always accompanied with the loss of metabolic process or with the loss of those enzymes sensitive to the drug, that is, with conversion of the metabolic pattern in the organisms. It is conceivable that the action of the antibiotic on sensitive strains may be due to its ability to react with the principal component of the metabolic system of α-ketoglutaric acid, whereas, this antibiotic has no or considerably less activity in the resistive strains in which the component of the metabolic system is so firmly bound to the essential enzyme as to compete successfully with this antibiotic. Saz et al.[52~53)] have recently investigated the properties of nitro reductase of *E. coli* sensitive or resistive to aureomycin and it has been indicated that in the development of resistance to

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32) M. G. Sevag and E. I. Rosanoff, *J. Bacteriol.*, **63**, 243 (1952).
33) N. Katunuma, T. Shoda and H. Noda, *J. Vitaminol.*, **3**, 77 (1957).

TABLE VIII. ACTION OF CHLORAMPHENICOL ON PRODUCTION OF α-KETOGLUTARATE FROM PYRUVATE, LACTATE AND ACETATE BY WASHED CELLS OF *E. COLI*

Substrates	Pyruvate plus acetate		*l*-Lactate plus acetate	
(mM)	1.600	2.000	2.000	2.000
Chloramphenicol added (mg/50 ml)	0	10	0	30
Washed cells of *E. coli* (mg)	300	300	100	100
Time of incubation (hours)	5	5	13	13
Consumption of C_3-acids (mM)	1.600	1.600	2.000	2.000
α-Ketoglutarate found (mM)	0.287	Trace	0.480	Trace

TABLE IX. ACTION OF CHLORAMPHENICOL ON PRODUCTION OF α-KETOGLUTARIC ACID FROM C_4-DICARBOXYLIC AND ACETIC ACIDS BY WASHED CELLS OF *E. COLI*

Substrates added	Fumarate plus acetate		Succinate plus acetate		DL-Malate plus acetate	
(mM)	2.000	2.000	2.000	2.000	2.000	2.000
Chloramphenicol added (mg/50 ml)	0	20	0	20	0	20
Dicarboxylic acids consumed (mM)	2.000	2.000	2.000	2.000		
α-Ketoglutaric acid found (mM)	0.603	Trace	0.650	0.205	0.560	Trace

aureomycin, an altered protein is synthesized which is able to bind essential cation firmly enough to compete successfully with aureomycin.

Action of Chloramphenicol

Considerable dissertations have been presented on the inhibiting action of chloramphenicol upon the metabolism of microorganisms. The following reactions are reported to be inhibited by chloramphenicol; the formation of adaptive enzyme[34~35] or the synthesis of protein[36~38], the activity of bacterial lipase[42], the reduction of nitrite[43], the metabolism of amino acid[39~41], and the oxidation of dicarboxylic acids[44~45].

It has already been demonstrated by the authors that the oxidative fermentations by bacteria of *coli-aerogenes* of C_3- and C_4-acids including pyruvic, lactic, succinic, fumaric, malic and oxalacetic acids give rise to a large amount of α-ketoglutaric acid[9]. Accordingly, it is now of interest to ascertain whether chloramphenicol would reveal any effect on the production of α-ketoglutaric acid by the bacteria of *coli-aerogenes*. Experiments were instituted with acetic, pyruvic and lactic acids in the same manner as in the experiments shown in Table I. Washed cells (100~300 mg as dry matter) of *E. coli* grown on glucose medium, were aerobically incubated in the presence or absence of chloramphenicol at 30°C for 5~13 hours on a media (50 ml) containing 5 mM phosphate buffer (pH: 6.0), 50 μM $MgSO_4$ and requisite amounts of substrates (Na-salts) as shown in Table VIII.

From the results of experiments given in Table VIII, it has been found that chloramphenicol remarkably inhibits the production of α-ketoglutaric acid from C_3 and C_2-acids by the bacterium. Similar experiments were carried with C_4-dicarboxylic acids such as fumarate, malate and succinate.

The results are shown in Table IX in which incubations were carried out in the presence or absence of chloramphenicol (at a level of 0~400 γ per ml of reaction mixtures) at 30°C for 14 hours on a media (50 ml) containing 5 mM phosphate buffer (pH: 6.0), 50 μM $MgSO_4$, 135 mg washed cells of *E. coli* grown on glucose medium and requisite amounts of Na-salts of C_4-dicarboxylic and acetic acids.

Very marked changes were shown again by a com-

34) F. E. Hahn and C. L. Wisseman, Jr., *Proc. Soc. Exptl. Biol. Med.*, **76**, 533 (1951).
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Table X. Influence of Chloramphenicol upon Oxidation of a Variety of Substrates by Washed Cells of *E. Coli*

Substrates (μM)	Bacterial cells (mg)	Time of incubation (min.)	Chloramphenicol (γ/2.3 ml)	O_2 uptake (μl)
Acetate 10	7	180	0	371
			1000	350
Pyruvate 10	7	180	0	410
			1000	438
dl-Lactate 80	7	120	0	1064
			1000	1086
Succinate 25	7	120	0	1000
			1000	976

parison between the oxidation of substrates by this organism in the presence and those in the absence of chloramphenicol (Table IX); α-ketoglutaric acid-fermentation occurs in the absence of this antibiotic, whereas, it has been found that the addition of the antibiotic to the reaction mixture brings about a striking decrease in the yield of α-ketoglutaric acid. Moreover, it should be noted that in this case as in experiments with C_3-acids there is no remarkable effect of chloramphenicol upon the oxidative decomposition of substrate to be observed. These observations were further ascertained by application of the Warburg technique in the following manner; the reaction mixture contained 200 μM phosphate buffer (pH : 6.5), 4 μM $MgSO_4$, amounts of the substrates (Na-salts) as indicated and 7 mg washed cells of *E. coli*, and in the centre well 0.2 ml 15% KOH; total volume 2.5 ml. Preincubation was carried out at 37°C in air for 60~180 minutes with or without chloramphenicol in the absence of substrates and thereafter, incubation was carried out in the presence of substrates. Table X shows that oxidation of C_2-, C_3- and C_4-acids by the organism grown on glucose, take place even in the presence of chloramphenicol.

Since dihydrostreptomycin was found to reveal an accelerating effect upon the oxidation of α-ketoglutaric acid by this organism, it is now a matter of importance to ascertain whether there is a similar effect of chloramphenicol upon the oxidative removal of α-ketoglutaric acid. However, it was found that the oxidation of α-ketoglutaric acid by the washed cells of *E. coli* was never accelerated by the presence of chloramphenicol. This indicates that the oxidative pathway in *E. coli* in the presence of chloramphenicol is of a different type from that brought about in the presence of dihydrostreptomycin. Thus, it has been suggested that chloramphenicol inhibits a reaction-system synthesizing α-ketoglutaric acid, while the action of dihydrostreptomycin (i.e. streptomycin) mainly stimulates the biological oxidation of α-ketoglutaric acid.

Action of Tetracycline

Ajl has investigated the effect of terramycin (oxytetracycline) and aureomycin (chlortetracycline) on the respiration of *E. coli* in the presence of various substrates, and it has been observed that these antibiotics exert a great inhibiting effect upon oxidation by the resting cells of *E. coli* of acetic, pyruvic and C_4-dicarboxylic acids, and also upon the metabolism of α-ketoglutaric and tricarboxylic acids[46].

In the present paper, the authors investigated the action of tetracycline and the related compounds such as aureomycin (chlortetracycline) and terramycin (oxytetracycline) upon the formation of α-ketoglutaric acid from various substrates by washed cells of *E. coli*. Incubations were carried out on a shaker at 30°C for a period of 4~13 hours in both the presence and absence of tetracyclines added, on a reaction mixture (40 ml) containing 5 mM phosphate buffer (pH : 5.5~6.5), 50~100 μM $MgSO_4$, amounts of Na-salts of various substrates as shown in Table X1 and 100~400 mg washed cells of *E. coli* prepared in the same manner as already mentioned.

From the results shown in Table XI, it was observed that tetracycline and the related compounds greatly inhibit the consumption of pyruvic acid by *E. coli* and therefore, the formation of α-ketoglutaric acid is greatly decreased under such conditions. These results were furthermore ascertained by application of the Warburg-manometer. From the results shown in Table XII it was observed that the bacterial oxidations of substrates

46) S. J. Ajl, *Bacteriol. Rev.*, **17**, 32 (1953).

TABLE XI. ACTION OF TETRACYCLINE AND RELATED COMPOUNDS UPON PRODUCTION OF α-KETOGLUTARATE BY WASHED CELLS OF *E. COLI*

Exp. No.	Time of incubation (hours)	Bacterial cells (mg)	Antibiotics added (mg/40 ml)	Substrates initial (mM)	Substrates consumed (mM)	α-Ketoglutarate found (mM)
I—a	13	130	0	Pyruvate 4.000	4.000	1.370
I—b	13	130	Oxytetracycline 7	Pyruvate 4.000	2.590	0.600
I—c	13	130	Tetracycline 10	Pyruvate 4.000	2.500	+
II—a	13	150	0	Pyruvate 4.000	4.000	0.768
II—b	13	150	Chlortetracycline 14	Pyruvate 4.000		Trace
III—a	13	150	0	*l*-Lactate 3.330 plus acetate 2.000		0.412
III—b	13	150	Chlortetracycline 14	*l*-Lactate 3.330 plus acetate 2.000		0
IV—a	13	100	0	Fumarate 2.000 plus acetate 2.000	Fumarate 1.650	0.246
IV—b	13	100	Oxytetracycline 10	Fumarate 2.000 plus acetate 2.000	Fumarate 0.830	Trace
IV—c	13	100	Tetracycline 10	Fumarate 2.000 plus acetate 2.000	Fumarate 0.530	0
V—a	4	400	0	Fumarate 2.000 plus acetate 2.000		0.274
V—b	4	400	Oxytetracycline 15	Fumarate 2.000 plus acetate 2.000		0
V—c	4	400	Tetracycline 15	Fumarate 2.000 plus acetate 2.000		0

such as pyruvate, acetate and fumarate were extremely inhibited by the presence of tetracyclines, when incubations were carried out for 70 minutes in the presence and absence of the antibiotics in air at 37°C with the media (2.5 ml) containing 150~300 μM phosphate buffer (pH: 6.0~7.8), 2~3 μM $MgSO_4$, definite amounts of Na-salts substrates and 5 mg of washed cells of *E. coli*, and in the centre well 0.2 ml, 15% KOH; total volume 2.5 ml.

DISCUSSION

It has already been reported that the oxygen uptake of cellular suspensions of some micro-organisms are increased by the presence of streptomycin[3,26,49]. Wasserman[8] found that streptomycin stimulated the total oxygen consumption of cellular suspensions of *E. coli*, when this organism oxidized a variety of substrates such as glucose, pyruvate, succinate, malate, glutamate and glycerol.

Wasserman suggests that streptomycin exerts the action in a manner similar to 2,4-dinitrophenol or sodium azide and therefore, the rapid cessation of division of the bacterial cell exposed

Table XII. Influence of Tetracycline upon Oxidation of Pyruvate, Acetate and Fumarate by Washed Cells of *E. Coli*

Substrates (μM)	Tetracycline (γ/2.3 ml)	Oxytetracycline (γ/2.3 ml)	O_2 uptake (μl)
none	0	0	50
Pyruvate			
50	0	0	750
50	1500	0	80
50	0	1500	145
Acetate			
60	0	0	534
60	1500	0	39
60	0	1500	30
Fumarate			
60	0	0	545
60	1500	0	27
60	0	1500	26

Preincubation for 60 min. at 37°C with or without tetracyclines in absence of substrates.

to streptomycin, should be related to the potency of the drug in uncoupling the assimilatory processes from oxidative dissimilation.

On the other hand, it has been concluded that the antibiotical action of streptomycin may be related more closely to nitrogen metabolism than to terminal respiration[1,2,3].

Experiments reported in this paper have revealed the fact that dihydrostreptomycin not only depresses the bacterial accumulation of α-ketoglutarate from a variety of carbonaceus substances including glucose, acetate, pyruvate, lactate and C_4-dicarboxylic acids such as succinate, fumarate, and malate, but also the drug stimulates the bacterial oxidation of α-ketoglutarate under suitable conditions. However, it should be remembered that the bacterial oxidations of substrates stated above such as C_2-, C_3- and C_4-acids, are never inhibited even under conditions in which dihydrostreptomycin is present. The authors have put forward an explanation that the antibiotical action of streptomycin may be related to the metabolism of α-ketoglutaric acid; this is to say that in the presence of the drug, α-ketoglutaric acid can not be metabolized towards the pathways for biosyntheses of glutamic acid and therefore, of the other varied amino acids, but its metabolism would proceed to complete oxidation. According to this explanation, it may be possible that the presence of streptomycin causes deficiency in the intracellular α-ketoglutaric acid of *E. coli*, so that the formation of adaptive enzyme (protein syntheses) in the organism tends to be depressed under such conditions.

Regarding the action of chloramphenicol, it has already been shown by Gale et al.[36] that the antibiotic exerts little effect on the intracellular accumulation of glutamic acid in *Staphylococcus aureus*, although it causes a very remarkable inhibition on the formation of combined glutamic acid, that is, the synthesis of protein is greatly inhibited by the presence of the drug at a leval of 0.03~1.5 m M concentration. Experiments reported in the present paper, show that chloramphenicol produces striking inhibition of the α-ketoglutarate-synthesizing system in *E. coli* without causing any inhibition on the oxidative removal of a variety of carbonaceus substances such as pyruvate, lactate and C_4-dicarboxylic acids. The results also indicate that there exist more than one metabolic process concerned in oxidation or the respiration system in the bacteria of *coli-aerogenes*; one is the α-ketoglutaric acid cycle sensitive to chloramphenicol, the other is a respiration system, by way of tricarboxylic acid, nonsensitive to chloramphenicol. It should be noted here that the major oxidation of tricarboxylic acid may occur under the action of isocitritase, although a part of oxidation may occur via α-ketoglutaric acid by the action of isocitric-dehydrogenase.

The formation of adaptive enzyme, that is, protein synthesis are well known to be greatly inhibited in the presence of chloramphenicol as well as in the presence of streptomycin. From the present investigation it has been suggested that the formation of an adaptive enzyme (protein synthesis) is inhibited in the presence of chloramphenicol, owing to the blockage of a α-ketoglutaric acid-synthesizing reaction. Tetracyclines inhibit the degradation of a variety

of carbonaceus substances such as glucose, acetate, pyruvate, lactate and C_4-dicarboxylic acids, so that α-ketoglutaric acid-fermentation is greatly inhibited in the presence of the drugs. It has already been reported that tetracyclines such as chlortetracycline and oxytetracycline exert an inhibiting effect on the phosphorylation system[47,48].

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Studies on Osmophilic Yeasts

Part IV. Change in Permeability of Cell Membranes of the Osmophilic Yeasts and Maintenance of their Viability in the Saline Medium

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A striking increase in permeability of cell membranes of *Sacchromyces rouxii*, a typical salt-tolerant yeast, occurred when they were cultivated in the saline medium. This change in permeability, however, did not occur in the concentrated sugar solution. It appeared that this increase in permeability should be attributed to the effect of sodium chloride itself, rather than to the osmotic effect. Therefore, this increase in permeability must be one of the most important points that serve to elucidate the mechanism of salt-tolerant property of yeast cells. The cells which had been changed in permeability by the effect of sodium chloride, could not maintain their viability in the saline medium unless an active metabolic process was allowed to proceed with the aid of the external substrates.

INTRODUCTION

In the previous paper[1], in which the nature of salt-tolerance and that of sugar-tolerance of the osmophilic yeasts were compared, it was found that the salt-tolerance and the sugar-tolerance are quite different. It therefore seemed that some factors other than the osmotic pressure

1) H. Ōnishi, This Bullesin, **21**, 137 (1957).

Table I. Contents of Potassium and Sodium of *Sacch. rouxii* Cultivated in the Concentrated Solutions of NaCl or Glucose

culture media	washing solutions	yeast dry matter 1g	
		K (mg)	Na (mg)
ordinary koji extract (bllg. 8)	distilled water	10.38	0.57
koji extract containing 18% NaCl	distilled water	0	0.47
	5% glucose water	0	0.96
	50% glucose water	0.57	0.94
	5% glucose 18% NaCl water	3.44	—
	5% glucose 18% KCl water	—	1.43
koji extract containing 50% glucose	distilled water	9.92	0.52

must be involved in the salt-tolerant mechanism of the osmophilic yeasts. Supposing that machanism of their salt-tolerance might mainly depend upon the environmental influence of the ions or the undissociated molecule of sodium chloride, the change of sodium and potassium contents of the cells in the saline medium was first observed in the present paper. A remarkable increase in permeability of cell membranes was found to occur in the saline medium, and the significance of this phenomenon in the salt-tolerant property of yeast cells is discussed.

EXPERIMENTAL AND RESULTS

[I] Increase in Permeability of Cell Membranes in the Saline Medium.

Although important roles of permeability of cell membranes in the salt-tolerant and halophilic microorganisms are naturally expected[2], very few investigations have hitherto been conducted from this view point. Conway et al.[3] prepared a "Na-yeast" in which all or nearly all the cellular potassium could be replaced by sodium, and the role of potassium and sodium in yeast physiology was investigated by comparing the property of such a yeast with those of the control potassium yeast. The mechanism of transport of potassium and sodium through cell membranes was discussed by Conway[4]. Takada[5] reported that a salt-tolerant mutant of *Sacchromyces ellipsoideus* showed an increased permeability to sodium as compared with the parent strain. Since studies on the characteristics of salt-tolerance of osmophilic yeasts in a high concentration of sodium chloride such as 18 percent have not been carried out from the standpoint of membrane-permeability to potassium and sodium ions, some experiments were conducted using *Saccharomyces rouxii* which is a typical salt-tolerant yeast[6] and is known to play an important role in soy-brewing.

(1) Potassium and Sodium Contents of *Saccharomyces rouxii* and the Change in Permeability of Cell Membranes when Cultivated in the Concentrated Solutions of Sodium Chloride or Sugars.

Method: Cells of *Sacch. rouxii* cultivated at 30° for 7~13 days in koji extract containing NaCl 0%, 18% or glucose 50%, were repeatedly centrifuged and washed three times with various washing solutions. The washed yeast cells were digested by boiling with conc. nitric acid, and the resulted solution was evaporated to a minimum volume to remove nitric acid and then diluted to an appropriate amount with distilled water. Analysis of potassium and sodium was carried out employing a Perkin-Elmer C-52 Flamephotometer. The results are shown in Table I.

It is very striking fact that yeast cells cultivated in the koji extract containing 18% sodium chloride showed a remarkable increase in permeability of potassium; almost all of the potassium in the yeast cells were lost by washing with distilled water. However, this complete loss of potassium in the cells did not appear to occur during growth in the high saline medium, because a considerable amount of potassium was still retained within the cells when they were washed with the solutions of the same osmotic pressure as that of 18% NaCl

2) W. L. Flannery, *Bact. Rev.*, **20**, 49 (1956).
3) E. J. Conway and P. T. Moore, *Biochem. J.*, **57**, 523 (1954).
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5) H. Takada and S. Tokuno, Lecture read at the meeting of the Botanical Society of Japan (October 12, 1957).
6) H. Ōnishi, This Bulletin, **21**, 151 (1957).

TABLE II. CHANGE IN PERMEABILITY WHEN THE CELLS OF THE ORDINARY CULTURE WERE SUSPENDED IN THE 18% NaCl PHOSPHATE BUFFER

period in suspending (hrs.)	glucose-free 18% NaCl phosphate buffer yeast dry matter 1g				5% glucose 18% NaCl phosphate buffer yeast dry matter 1g			
	K (mg)		Na (mg)		K (mg)		Na (mg)	
	washed by dist. water	washed by 18% NaCl water	washed by dist. water	washed by 18% KCl water	washed by dist. water	washed by 18% NaCl water	washed by dist. water	washed by 18% KCl water
0	11.2	—	0.3	—	11.2	—	0.3	—
1	3.9	—	1.3	—	3.6	—	1.5	—
3	3.6	—	1.6	—	3.6	—	1.6	—
24	0	3.4	1.6	—	0	3.8	0.6	2.6
48	0	1.0	0.7	2.7	0	2.6	0.4	1.9

solution. The content of sodium in the cells remained unchanged in this washing, indicating that sodium ions do not enter into the cells in place of the extruded potassium. When these cells were washed with a 5% glucose solution, which should serve as the energy source to this yeast, potassium was washed out similarly as in the case of washing with distilled water. On the contrary, this change in permeability of cell membranes could not be recognized in the cells grown in the 50% glucose medium, which had the same osmotic pressure as that of 18% NaCl.

(2) Change in Permeability when Yeast Cells grown in the Ordinary Culture Medium were Suspended in a Phosphate Buffer Containing 18% NaCl.

Method: Cells of *Sacch. rouxii* cultivated in ordinary koji-extract at 30° for 7 days were washed twice with distilled water by centrifugation, and the washed cells were then suspended in the M/15 phosphate buffer solution (pH 4.8) containing 18% NaCl with or without the added glucose, and the suspensions were incubated at 30°. Cells taken at each interval were washed and analysed for potassium and sodium as similarly as described in (1). The results are shown in Table II.

From Table II, it is clear that the increase in permeability of the cells without the process of cell multiplication could be fully observed when the cells of the oridnary culture were suspended in the 18% NaCl phosphate buffer for 24 hours at 30°, and in this case any significant difference in the process of permeability change could not be recognized between the two suspending media with and without added glucose (5%).

However, a significant difference was found in the viability of the yeast cells between the above two environments with and without glucose; a remarkable loss in viability occurred in the glucose-free 18% NaCl phosphate buffer whereas, in the 18% NaCl phosphate buffer with added glucose (5%) no loss or only a slight loss of viability took place. (Fig. 1).

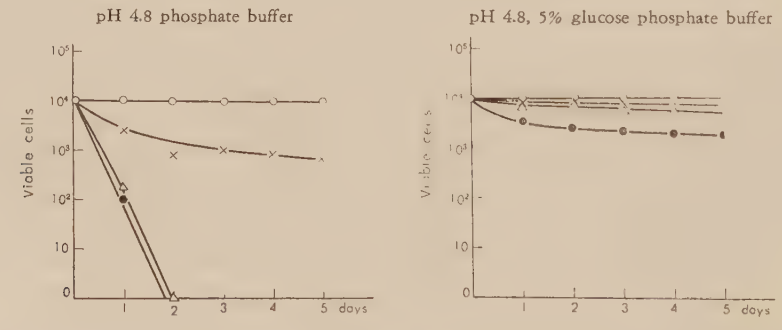


FIG. 1. Effect of Glucose on the Maintenance of Viability in the Concentrated Solution of NaCl (1).

(When the cells of the ordinary culture were transferred to the saline medium)

○: NaCl 0, ×: NaCl 1 mol, △: NaCl 2 mol, ●: NaCl 3 mol.

When cells of the ordinary culture were suspended in the phosphate buffer containing sodium chloride of a relatively low concentration such as 5%, the increase in cell permeability was also recognized as shown in Table III.

Table III. Change in Permeability when the Cells of the Ordinary Culture were Suspended in the 5% NaCl Phosphate Buffer

washing solutions	yeast dry matter 1g	
	K (mg)	Na (mg)
distilled water	0.63	1.10
5% glucose 5% NaCl water	5.51	—
5% glucose 5% KCl water	—	1.49

(3) Effect of Temperature on Change in Permeability of Yeast Cells in the Saline Medium.

When the cells of *Sacch. rouxii*, cultivated in koji extract, were suspended in the 18% NaCl phosphate buffer with or without glucose and kept in a refrigerator (at 0°~5°), any change in permeability could not be observed even after a long period of storage, unlike those in the case of 30°. This phenomenon was similarly found to occur without regard to the addition of glucose. The results are shown in Table IV.

Table IV. Effect of the Low Temperatures on Change in Permeability of *Sacch. rouxii* in the Saline Medium

period in suspending (days)	glucose-free 18% NaCl phosphate buffer yeast dry matter 1g		5% glucose 18% NaCl phosphate buffer yeast dry matter 1g	
	K (mg)	Na (mg)	K (mg)	Na (mg)
0	10.61	0.66	10.61	0.66
1	5.60	3.76	5.60	3.47
5	2.13	3.54	1.76	3.24

In this case, it was also noted that viability of the cells of the 18% NaCl medium was maintained without the addition of glucose to the suspending medium (Fig. 2).

It was further observed that loss in viability rapidly occurred when the cells of *Sacch. rouxii*, cultivated in the 18% NaCl medium, were transferred to the glucose-free 18% NaCl phosphate buffer, though the loss of viability of cells was tolerably prevented by the supply of glucose. (Fig. 3).

From the above facts, it was presumable that the viability of *Sacch. rouxii* in the concentrated solutions of sodium chloride was maintained without any supply of glucose under the conditions in which no increase in cell permeability could occur, but no matter how

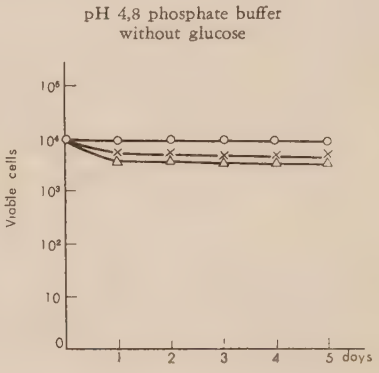


Fig. 2. The Maintenance of Viabitity in the Saline Medium at the Low Temperature of 0°~5°.

○: NaCl 0, ×: NaCl 10%, △: NaCl 18%,

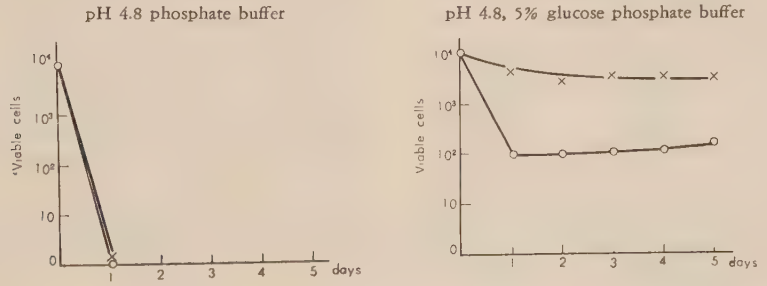


Fig. 3. Effect of Glucose on the Maintenance of Viability in the Concentrated Solution of NaCl (2).

(When the cells of the 18% NaCl culture were transferred to the saline medium)

○: NaCl 0% phosphate buffer ×: NaCl18% phosphate buffer

sufficiently they might be adapted toward sodium chloride, the cells, which had an increased cell permeability in the saline medium, would need some active metabolism by utilizing the energy source such as glucose for the maintenance of their viability in the concentrated solution of sodium chloride.

[II] The Relation between the Acquirement of the Salt-tolerance by the Osmophilic Yeasts and the Increase in Permeability of the Cell Membranes.

The growth curves of *Sacch. rouxii* in the 18% NaCl medium when they were precultured in the media containing different NaCl concentrations, were examined in the previous paper[1]. From the results, it could be said that when the yeast cells grown in a medium of a relatively low NaCl concentration such as 5% NaCl were transferred into the 18% NaCl medium, the loss of viability was remarkably slight, indicating the acquirement of a sufficient salt-tolerant property in such a low NaCl medium.

Considering the distinct increase in permeability by suspending in the 5% NaCl medium as shown in Table III and the growth curve reported in the previous paper[1], adaptive process of *Sacch. rouxii* toward the high sodium chloride concentration might be involved in some active metabolism resulting from the increase in permeability.

The cells which were suspended in the 18% NaCl medium at a low temperature, did not increase their permeability as illustrated in Table IV. When such cells were transferred to the 18% NaCl medium and incubated at 30°, a remarkable fall in viability was observed. (Table V)

Table V. Fall in Viability when the Cells, which were Suspended in the 5% Glucose 18% NaCl Phosphate Buffer at 0°~5° for 3 days, were Plated on the 18% NaCl Koji Agar

period in suspending (days)	NaCl 0% koji agar	18% NaCl koji agar
0	10848	1920
1	5088	788
3	1200	512

When *Sacch. rouxii* cells from the ordinary culture were transferred to the concentrated sugar solution which had the same osmotic pressure as that of 18% NaCl solution, no fall in cell viability was recognized. (Fig. 4). In this case, no change in permeability occurred as shown in Table I.

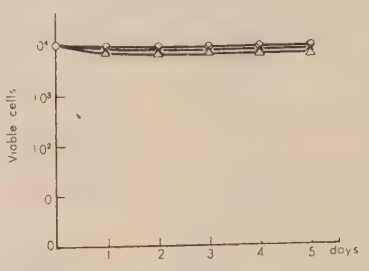


Fig. 4. The Maintenance of Viability in the Concentrated Solution of Sugar.

○: phosphate buffer
×: 50% glucose phosphate buffer
△: 40% rhamnose phosphate buffer

From these facts, it became much more apparent that the difference in the characteristics of salt-tolerance and sugar-tolerance of the osmophilic yeasts could be attributed to the differences in permeability of cell membranes in the concentrated solution of salts or sugars.

When the cells of *Sacch. rouxii*, precultured in the 50% glucose koji extract were plated on the agar media containing 50% glucose and 18% NaCl, colonies of almost the same numbers were obtained in both media. (Table VI)

It was noted that loss in viability did not occur by transferring the cells from the 50% glucose medium to the 18% NaCl medium, but any increase in permeability of the cells grown in 50% glucose medium was found not to occur as illustrated in Table I. And then, when the cells cultivated in the 50% glucose medium were transferred to the 18% NaCl medium, a similar increase in cell permeability was observed. (Table VII).

When washed cells of the ordinary culture were

Table VI. The Salt-tolerant Property of the Cells of the Glucose 50% Culture

strains	50% glucose koji agar	18% NaCl koji agar	strains	50% glucose koji agar	18% NaCl koji agar
1	812	720	15	1688	1800
2	1240	1128	28	1296	1336
5	1352	1232	31	1224	1296
8	1528	1512	34	792	844

Table VII. Change in Permeability when the Cells of the 50% Glucose Culture were Suspended in the 5% Glucose 18% NaCl Phosphate Buffer

period in suspending (days)	washing solution	yeast dry matter 1g	
		K (mg)	Na (mg)
0	distilled water	6.08	0.53
1	distilled water	0	0.80
	5% glucose 18% NaCl water	1.27	—
	5% glucose 18% KCl water	—	1.67

suspended in the 50% rhamnose phosphate buffer at 30° for 24 hours and then transferred to the 18% NaCl medium, a fall in viability was obviously observed.

Thus, the fact that yeast cells which cultivated in the 50% glucose medium can easily acquire the salt-tolerant property, may probably be explained as follows: an active metabolic process may be needed for the maintenance of the viability as a result of increased permeability of the cell membranes which occurred when the cells were transferred from the ordinary culture to the 18% NaCl medium. During growth in the 50% glucose medium, the cellular organization, by which this essential metabolic process is allowed to proceed very smoothly, may be built up. This fact was not attributable to the lack of osmotic shock when the cells were transferred between the two different media of the same osmotic pressure.

[III] Effect of Various Substances on Maintenance of Viability of the Cells in the Concentrated Solution of Sodium Chloride.

It was shown in section [I] that supply of glucose, which was available as the energy source to this yeast was essential for the maintenance of viability of the yeast cells in the saline medium. The effects of various other substances such as sugars, organic acids and amino acids were examined. The washed cells grown in the ordinary medium were suspended in the NaCl-free or 18% NaCl phosphate buffer (pH 4.8) to which the various substances had been added, and the cell supensions were incubated at 30°. The number of viable cells was determined by plating on koji agar. As shown in Fig. 5, pyruvate, acetate, succinate, citrate, *dl*-α-alanine, *l*-glutamic acid and lactose were not effective. Even under vigorous aeration, these added substances showed no effect.

DISCUSSION AND CONCLUSION

Conway et al.[3] showed that in "Na-yeast", 98% of the cellular potassium of the yeast is replaced up by the sodium and that the replaced ion could not be swept away by washing the cells with distilled water. It seems to be noteworthy that this phenomenon is merely an exchange of Na^+ and K^+ but not an increase in permeability of cell membranes. By measuring the cellular potassium and sodium contents of *Sacch. rouxii*, the present author found that permeability of cell membrances of *Sacch. rouxii* in the concentrated solution of sidium chloride is remarkably increased and consequently, almost all of cellular potassium is lost by washing with distilled water. This would be quite a different phenomenon from that of "Na-yeast."

Since permeability of cell membranes of *Sacch. rouxii*, which was cultivated in the 50% glucose medium of the same osmotic pressure as 18% NaCl remained unchanged, it became much more clear that an increase in cell permeability could not take place merely by an osmotic effect but this may be due to the effect of sodium chloride itself. Therefore, the author is of the opinion that this increase in permeability must be one of the most important points for elucidation of the mechanism of salt-tolerance.

As stated above, the cells of *Sacch. rouxii* which easily lose their cellular potassium in the saline medium, do not permit the entrance of sodium into the cell in exchange for potassium. The increase in permeability occurred by suspending the cells in the saline medium either with or without added glucose, but in the glucose-free saline medium the yeast cells rapidly die out. Moreover, when the cells which were

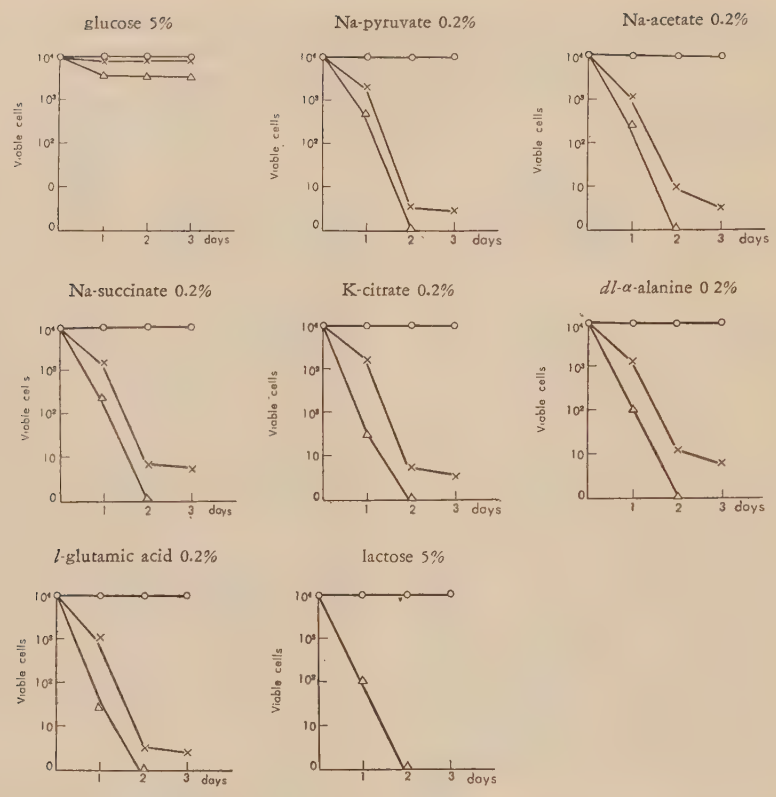


FIG. 5. Effect of Various Substances on the Maintenance of the Viability of the Cells in the Concentrated Solution of NaCl.
○: NaCl 0%, ×: NaCl 10%, △: NaCl 18%

cultivated in the 18% NaCl medium and thus fully adapted toword the high sodium chloride environment, were transferred to the glucose-free saline medium, they immediately died out. This seems to indicate that the cells the permeability of which have been changed by the action of sodium chloride can not maintain their viability unless an active metabolic process is allowed to proceed with the aid of an external substrate such as glucose.

The easy loss of cellular potassium of *Sacch. rouxii* which results from the effect of sodium chloride is of special interest since it is known that potassium plays an important role in yeast metabolism: Scott et al.[7] studied the influence of glycolytic inhibitors on the potassium and sodium contents of baker's yeast showing that glycolytic factors are involved in potassium retention. Schmidt et al.[8] observed that the absorption of orthophosphate and the formation of metaphosphate in baker's yeast cells accompanied by the absorption of cation with a strong preference for potassium. Goodman and Rothstein[9] pointed out that glycolysis and potassium ion play an important role in the uptake of phosphate by baker's yeast. Meyerhof and Kaplan[10] stated that fermentation is completely

7) G. T. Scott, M. A. Jacobson and M. E. Rice, *Arch. Biochem.*, **30**, 282 (1950).

8) G. Schmidt, L. Hecht and S. J. Thannhauser, *J. Biol. Chem.*, **178**, 733 (1949).

9) J. Goodman and A. Rothstein, *J. Gen. Physiol.*, **40**, 915 (1957).

10) O. Meyerhof and A. Kaplan, *Arch. Biochem. and Biophys.*, **33**, 282 (1951).

absent without either K^+ or NH_4^+ and without Mg^{++}.

The effect of this tendency of easy loss of potassium on the metabolism of the salt-tolerant yeasts and its physiological significance should be further investigated in detail.

It was further noted that a change in permeability of cell membranes of *Sacch. rouxii* which was cultivated in the concentrated solution of glucose could not be observed. However, it seemed that in this case, some intracellular metabolic process different from that in the ordinary culture, may prevail supposing that this will be effective for prevention of the loss in viability when this yeast is transferred to the saline medium. In order to clarify the cause of this phenomena, intracellular metabolism of *Sacch. rouxii* in the three comparative environments, the ordinary medium, the concentrated glucose medium and the high salt medium, must be carried on further.

Acknowledgement I would like to express my sincerest thanks to Emeritus Prof. K. Sakaguchi of University of Tokyo for his kind guidance and encouragemt throughout the course of this work. Great indebtness is also acknowledged to Prof. T. Asai and Prof. Y. Sumiki for their valuable suggestions. I also wish to thank Dr. R. Ishida of the Government Chemical Industrial Research Institute, Tokyo for his kind guidance in the flamephotometric analysis and Dr. M. Mogi, the Director of Noda Institute for Scientific Research for his interest and encouragement and Mr. A. Kaneko for his willing help at all times.

Separation and Crystallization of Each Component from Crystalline Phosphoglyceric Acid Mutase

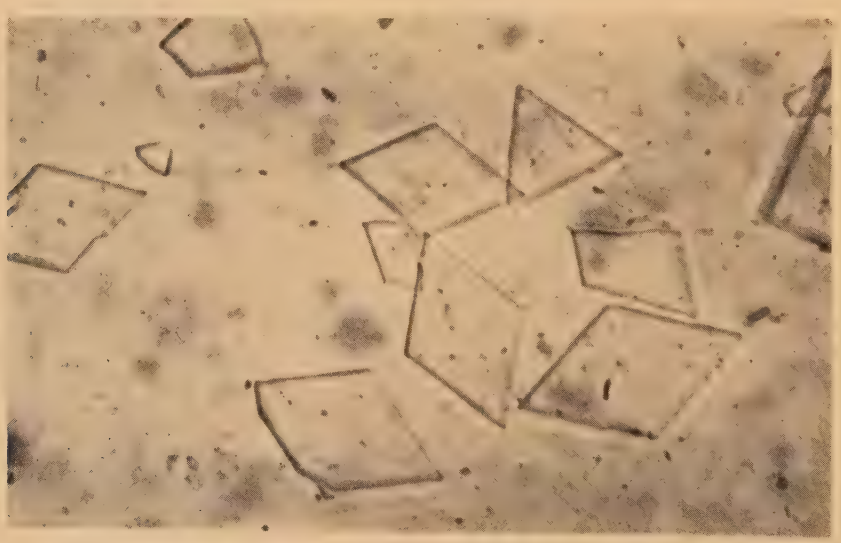
Sir:

Crystalline yeast phosphoglyceric acid mutase has been found to be composed of several components by means of electrophoretic analysis[1,2]. We have also found that each component separated by horizontal zone electrophoresis is the phosphoglyceric acid mutase protein itself and has a different specific activity[2]. But in order to study the differences among the individual components, it was necessary to isolate each component in large quantities. In the case of horizontal zone electrophoresis, it was difficult to apply a large amount of protein.

We have succeeded in separating a large amount of each component by using the special vertical zone electrophoresis apparatus with Hg-calomel electrodes, which is deviced to compensate electroosmosis automatically. The supporting material used in this apparatus was cellulose powder prepared by methanolising good quality clean cotton[3]. About 300 mg of the crystalline phosphoglyceric acid mutase protein was applied on the column and zone electrophoresis was carried out at 6°C in a veronal buffer of pH 7.0 and 0.065 ionic strength for 150 hours at 40 mA with 240 volts applied over the apparatus.

The specific activity of each component thus separated was as follows: component I, 4,060; II, 3,630; III, 2,240 and IV, 200, respectively. (The enzyme activity was directly measured by the polarimetric method[4].) Moreover, we have also succeeded in crystallization of each component, which was achieved by dropwise addition

1) H. Edelhoch, V. W. Rodwell and S. Glisolia, *J. Biol. Chem.*, **228**, 891 (1957).
2) H. Chiba and E. Sugimoto, This Bulletin, **23**, 213 (1959).
3) P. Flodin and D. W. Kupke, *Biochim. et Biophys. Acta*, **21**, 368 (1956)
4) H. Chiba and E. Sugimoto, This Bulletin, **23**, 207 (1959)



Component I

of saturated ammonium sulfate solution at 0°C. Crystalline forms of the individual components were the same.

The photograph shows an instance representing the crystalline component I with the highest specific activity.

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Studies on Lignin

Part VII. Isolation and Identification of 2-Ethylthio-1-(4-hydroxy-3-methoxyphenyl)-propanone-(1) from Mercaptolysis Oil of Pine Ethanol Lignin

Sir:

As it has been considered that the initial stage of chemical reactions occurring in the Kraft process is the formation of benzyl mercaptans from benzyl groups contained in lignin and inorganic sulfides in the cooking solution[1,2], it seemed to be of interest to study the reaction of mercaptan with lignin not only in alkaline solution but also in neutral or acid media. Recently, Gierer carried out the cooking of lignin with ethyl mercaptan in neutral solution, and found that an appreciable amount of the mercapto group is combined with lignin[3]. On the other hand Holmberg, as early as thirty years ago, introduced the method of mercaptolysis into lignin chemistry, and showed that the mercaptan lignin contained almost one sulfur atom per methoxyl[4]. However, investigations on the low molecular fraction of the mercaptolysis product have not yet been carried out. We have studied on the petroleum ether-soluble oil obtained from mercaptolysis of lignin.

Pine ethanol lignin[5] was cooked with a mixture of ethanol and ethyl mercaptan (4:1 vol.) at 100° for 15 hours in an autoclave using 2% hydrogen chloride as a catalyst. After neutralization with sodium bicarbonate, the concentrated solution was poured into a large amount of petroleum ether. The phenol fraction of the petroleum ether soluble oil (4~5% of lignin) was passed through a magnesol column using benzene as the solvent. The first fraction (about 1% of lignin) was converted into *p*-nitrobenzoate in the usual manner. After having been washed with ether and recrystallized twice from ethanol, the product melted at 139.5~141°. The yield obtained was about 0.2% of lignin as *p*-nitrobenzoate. *Anal.* Calcd. for $C_{19}H_{19}O_6SN$: C, 58.61; H, 4.92; S, 8.22; N, 3.61. Found: C, 58.78; H, 5.11; S, 7.86; N, 3.77. This was identified by mixed melting point determination (m.p. 141~142.5°) with a synthetic specimen as *p*-nitrobenzoate of 2-ethylthio-1-(4-hydroxy-3-methoxyphenyl)-propanone-(1) (I), which is a thioether corresponding to one of Hibbert's ketol ethers (II)[5].

I R–CO–CH(SEt)–CH_3
II R–CO–CH(OEt)–CH_3
III R′–CO–CHBr–CH_3
R=4-hydroxy-3-methoxyphenyl
R′=4-acetoxy-3-methoxyphenyl

Absorption around 300 mμ in U. V. spectrum

1) T. Enkvist and M. Moilanen, *Svensk Papperstidn.*, **52**, 183 (1949).
2) H. Mikawa, K. Sato, C. Takasaki and K. Ebisawa, *Bull. Chem. Soc. Japan*, **29**, 265 (1956).
3) J. Gierer and B. Alfredsson, *Acta Chem. Scand.*, **11**, 1516 (1957).
4) cf. E. Hägglund: "Chemistry of Wood", Academic Press, New York, 1951, p. 252.
5 A. B. Cramer, M. J. Hunter and H. Hibbert, *J. Am. Chem. Soc.*, **61**, 509 (1939).

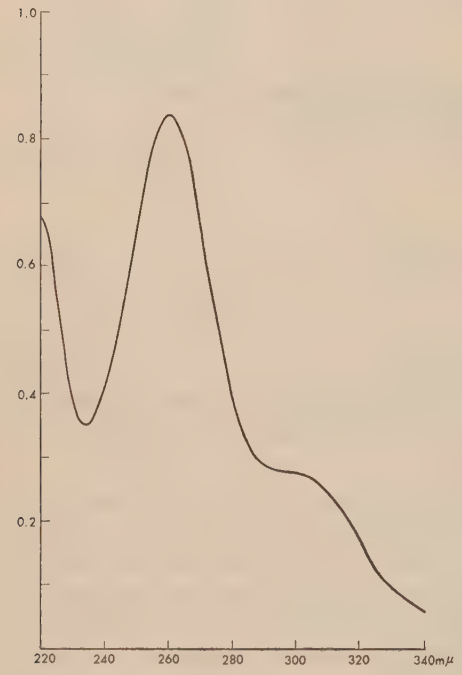

Fig. 1

(Fig. 1) is interpreted as an indication of the presence of the carbonyl group at α-position of the side chain, since *p*-nitrobenzoate of vanillyl ethyl sulfide[6] has no absorption around this region.

The synthesis of (I) was conducted by a reaction of 2-bromo-1-(4-acetoxy-3-methoxyphenyl)-propanone-(1) (III)[7] with two mols of sodium ethyl mercaptide in ethanol solution at room temperature. The first one mol reacted with brom to form thioether, and the second was consumed in saponification of the acetyl group. (I) obtained as a yellow oil was converted into *p*-nitrobenzoate. After having been recrystallized twice from ethanol the product melted at 143~143.5°. *Anal.* Calcd. for $C_{19}H_{19}O_6SN$: C, 58.61; H, 4.92; S, 8.22; N, 3.61. Found: C, 58.54; H, 4.83; S, 7.76; N, 3.44. The U. V. spectrum was comletely identical with that of *p*-nitrobenzoate obtained from mercaptolysis oil of lignin. Experimental details concerning the isolation and synthesis of (I) will be published later.

It is a well-known fact that carbonyl groups readily react with mercaptan to form mercaptole or mercaptal in the presence of mineral acid. It was, therefore, contrary to our expectation that a compound carrying the carbonyl group had been isolated from mercaptolysis oil.

It is conceivable that (I) originated from (II) which may have been contained in ethanol lignin used as the starting material, and did not originate from lignin itself. In order to clarify this point, further studies are now in progress.

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6) J. Gierer and B. Alfredsson, *Chem. Ber.*, **90**, 1240 (1957).

7) B. Riegel and H. Wittcoff, *J. Am. Chem. Soc.*, **68**, 1913 (1946).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 4, p. 342~343, 1959]

Isolation of Isomaltose from Honey

Sir:

We have previously reported[1] that kojibiose is isolated from honey (*Lespedeza bicolor*) by the application of carbon-Celite column and Magnesol-Celite column chromatographic procedures. On the other hand, Goldschmidt et al.[2] detected isomaltose in honey by paper chromatography.

We now report on the isolation and identifica-

1) T. Watanabe and K. Aso, *Nature*, a forthcoming publication.

2) S. Goldschmidt and H. Burkert, *Hoppe-Seyler's Z. Physiol. Chem.*, **300**, 188 (1955).

tion of isomaltose. Honey, 300 g was fractionated on a carbon-Celite column (550×125 mm) using water (22 l), 2.5% (22 l), 5% (14 l), 10% (18 l), 15% (18 l), 20% (14 l), 25% (12 l) and 30% (30 l) concentrations of ethanol as successive elution solvents. Isomaltose was detected in 2.5~5% ethanol effluent by paper chromatography. The effluent portions containing isomaltose from three columns (900 g of honey) were combined (16.5 g of dried material). These portions were contaminated with a considerable amount of kojibiose, two oligosaccharides containing ketose, glucose, fructose, sucrose and a trace of leucrose.

The sugar mixture was then rechromatographed on a carbon-Celite column (530×80 mm) applying the gradient elution method with 0~3.0% aqueous ethanol containing borate buffer (pH 10.0)[3]. The 0% ethanol fractions contained monosaccharides, and 0.5~1.5% ethanol fractions were composed of isomaltose and several sugars containing ketose. By the removal of borate from the sugar complex by repeated distillation with methanol, 7.2 g of sugar mixture (white amorphous powder) was obtained. A portion of 2.1 g of this white amorphous powder was acetylated as usual to give 3.1 g of crude acetate. Direct crystallization from ethanol was unsuccessful. One and a half g of this crude acetate was dissolved in 10 ml of benzene and poured on to a column (300×40 mm) of Magnesol-Celite (5:1) and developed with 1000 ml of benzene: *t*-butanol (100:1, by volume). A zone which appeared at 122~174 mm from the top of the column by means of potassium permanganate streak indicator was sectioned from the column and eluted with acetone. Removal of the solvent left 0.3 g of syrup, from which 37 mg of fine prisms was obtained upon crystallization from ethanol. After recrystallization, it had m.p. 144~145°, undepressed on admixture with an authentic β-isomaltose octaacetate.

We wish to acknowledge our indebtedness to Mr. K. Matsuda for his kind advice.

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3) S. A. Barker, E. J. Bourne and O. Theander, *J. Chem. Soc.*, **1955**, 4276.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 4, p. 343~344, 1959]

The Occurrence of Gibberellin A_1 in Water Sprouts of Citrus

Sirs:

In recent years, a number of reports[1,2,3] have been published indicating that gibberellins, or closely related substances occur naturally in higher plants, regulating the growth promoting system. In this communication, the authors wish to report on the isolation and identification of gibberellin A_1 from the elongated shoots of *Citrus Unshiu*. It is specially worthy of note that gibberellin A_1 exists in the shoots of a tree. Extraction and purification procedures were carried out by the following method.

Young water sprouts of *Citrus Unshiu* (mandarin orange) (7.2 kg.) were taken off their leaves, cut, ground by the blendor, immersed in 50% aqueous acetone (10 l.) and set alone overnight at room temperature. After removal of the residue by filteration, the eluate was evaporated to a small volume under reduced pressure, adjusted to pH 8.0 with sodium bicarbonate, and extracted with ethylacetate.

1) J. W. Mitchell, et al.: *Science*, **114**, 159 (1951).
2) B. O. Phinney, et al.: *Proc. Natl. Acad. Sci.*, **43**, 398 (1957).
3) J. MacMillan, et al.: *Naturwiss.*, **45**, 46 1958).

The aqueous layer was acidified to pH 3.0 with dil. sulfuric acid, and again extracted with ethylacetate, which was dried with anhydrous sodium sulfate. After evaporating the solvent, a dark greenish oil was obtained. For the purification of a gibberellin-like factor from this substance, the counter current distribution method (15 plates, ethylacetate/pH 5.2 phoshate buffer) was applied and the active fractions (plates No. 5–11) were collected and evaporated (1.2 g.). Then it was poured onto a column of cellulose powder (Whatman) and eluted with iso-propanol—28% ammonia—water (10 : 1 : 1). The active fractions were combined, evaparated (98 mg.), spotted on Whatman No. 1 filter paper and developed by the ascending method with the solvent system described above. As the R_F value of the biologically active substance closely coincided with that of gibberellin A_1 or A_3 (0.45 and 0.47, respectively), the areas, detected by test spots of an authentic specimen on both sides of the paper sheets, were cut out and eluted with methanol.

In order to change ammonium salt to free acid, the eluate was passed through a short column of Amberlite IR-120 resin (H^+ form). On evaporation of the solvent under reduced pressure, colorless amorphous powder was obtained (12 mg.), which on recrystallization from ethanol-ethylacetate-ligroin, gave colorless prisms (2 mg.) and melted at 230~6°C (decomposition) on Koffler's block. Its infrared absorption spectrum of a Nujol mull was completely identical with gibberellin A_1. The methyl ester of this substance, prepared with diazomethane also showed identical infrared absorption bands with those of an authentic specimen.

From this evidence and another report[3], it seemed most probable that at least, gibberellin A_1, is participating either directly or indirectly in the growth regulating system of higher plants.

Biological activity at each step was assayed by means of rice seedlings which is now employed in the authors' laboratory.

The authors wish to express their thanks to Dr. K. Furusato for kindly supplying the material used here.

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Biochemical Studies on "Bakanae" Fungus. Part 54
Chemical Structure of Gibberellins. Part. XX

Sirs:

As previously reported[1,2,3], the chemical structure of gibberellin A_1 was presented as the formura (I) and the position of the tertiary hydroxyl group of gibberellin A_2 was confirmed to be the C-15 of the ring D, and thus the partial structural formulae of A_2 and A_4 were proposed. In this communication, we wish to report on the structural relation between A_1 and A_2 and to present the final structure of gibberellins A_2 (II) and A_4 (III).

When monoacetyl A_2 methyl ester (II-a) was treated vigorously with phosphorus oxychloride and pyridine, its tertiary hydroxyl group was

1) Y. Seta, N. Takahashi, H. Kitamura and Y. Sumiki, This Bulletin, **22**, 429–431 (1958).
2) N. Takahashi, Y. Seta, H. Kitamura and Y. Sumiki, *ibid.*, **22**, 432–3 (1958).
3) H. Kitamura, N. Takahashi, Y. Seta and Y. Sumiki, *ibid.*, **22**, 434–5 (1958).

dehydrated to yield a product (III-a) (yield: 60%), $C_{22}H_{28}O_6$, m.p. 130~1°, and a mixture (yield: 40%) of two substances (III-b) and (III-c), through the adsorpto-chromatography on alumina. The fact that the product (III-a) is a same compound as monoacetyl A_4 methyl ester has already been published[3].

This fact has been conclusively proved by the ozonolysis of the above dehydrated products as follows. In the case of the former product (III-a), the ozonolysis furnished formaldehyde and a five-ring ketone (IV-a), $C_{21}H_{26}O_7$, m.p. 189~190°, infrared absorption at 1780 cm^{-1} (lactone), 1757 cm^{-1} (5-ring carbonyl), 1735 cm^{-1}

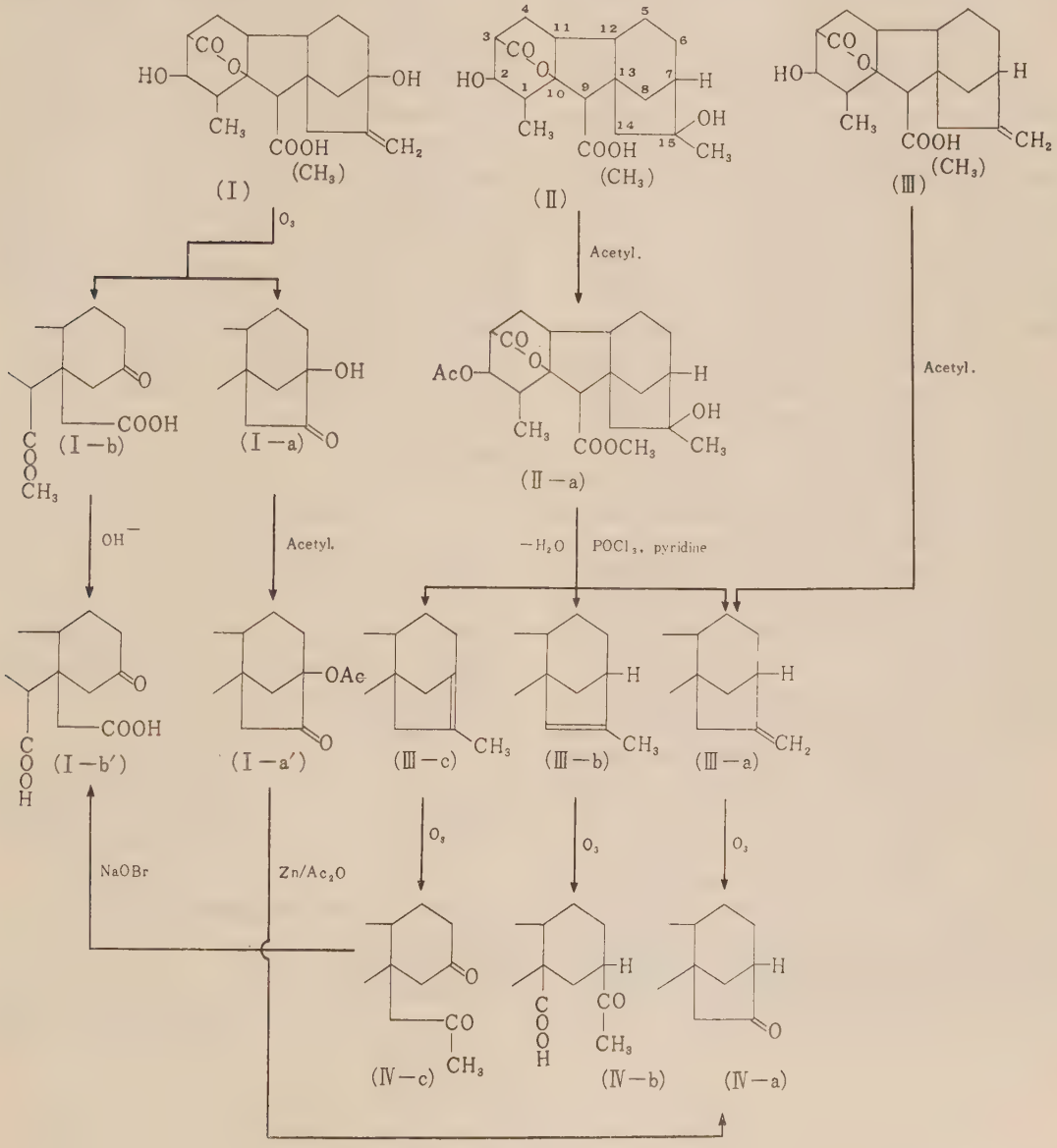

FIG. 1.

(carbomethoxyl and acetyl carbonyl), elucidating the existence of the five-membered exocyclic methylene in the dehydrated product, (III-a). While the latter mixture gave a ketonic acid (IV-b), $C_{22}H_{28}O_9$, m.p. 250~2° (decomp.) (iodoform reaction: positive) and a neutral keto-compound (IV-c), $C_{22}H_{28}O_8$, m.p. 204~5° (iodoform reaction: positive), infrared absorption at 1757 cm^{-1} (lactone), 1730 cm^{-1} (6-ring carbonyl), 1721 cm^{-1} (ester and saturated open-chain ketone), this result shows that the positions of the C-C double bond of dehydrated products (III-b) and (III-c) should be at C-14~C-15 and C-7~C-15, respectively. Moreover, the oxidation of the above neutral keto-compound (IV-c) with sodium hypobromite gave a dicarboxylic acid, $C_{18}H_{22}O_8$, m.p. 93~5° (decomp.) (its methyl ester m.p. 165°), after purification by the partition chromatography on silicic acid, which was proved to be identical with the product (I-b′) obtained from hydrolysis of an acidic product (I-b) which was yielded by ozonolysis of A_1[4], by mixed melting point and infrared spectra comparison. The identity of the above two compounds confirms that the secondary hydroxyl, lactone and carboxyl groups of A_2 are located at the same positions as those of A_1. Therefore, the final structural formulae of gibberellins A_2 and A_4 should be (II) and (III), respectively. Further evidence in support of the structure of A_2 assigned to (II) was furnished by the deacetoxylation (boiling at 145° for 36 hrs. with zinc and acetic anhydride) of the diacetyl compound (I-a′), obtained by acetylation of α-ketol compound (I-a) which was prepared by ozonolysis of A_1.

Further evidence indicating that the attaching position[2] of lactone alkyl oxygen is the C-10 as shown in Fig. 1, was given from the following experiments. By dehydration with phosphorus oxychloride in pyridine, both the secondary and tertiary hydroxyl groups of A_2 methyl ester were simultaneously dehydrated to give a dianhydro-derivative, $C_{20}H_{24}O_4$, m.p. 145~6°, which afforded a tetrahydro-anhydro-derivative, $C_{20}H_{28}O_4$, m.p. 167~8°, by catalytic reduction absorbing two moles of hydrogen. When the tetrahydro-dianhydro-derivative was treated with an excess of lithium aluminum hydride in boiling tetrahydrofuran for 24 hrs, were obtained two reduction products, diol $C_{20}H_{32}O_4$, m.p. 200~2° and triol $C_{19}H_{32}O_3$, m.p. 169~170°. The former diol, is the reduction product of only the lactone group of the tetrahydro-dianhydro-derivative and the latter triol, the reduction product of both lactone and carbomethoxyl groups. When each reduction product was acetylated with acetic anhydride and pyridine at room temperature for 48 hrs, a monoacetyl derivative (syrup) was obtained from the former diol, and a diacetyl derivative $C_{23}H_{36}O_5$, m.p. 201~2°, from the latter triol. These two acetyl derivatives showed an OH band at 3510 cm^{-1} and moreover, when the diacetyl derivative was oxidized with chromic acid, the original substance was recovered. Therefore, the hydroxyl group derived from lactone oxygen should be tertiary.

As previouly reported, the structural relation between A_2 and A_4 was determined already[3]. Therefore, the chemical structure of A_2 has been confirmed and therefore that of A_4 should be the formula (III).

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4) Y. Seta, H. Kitamura, N. Takahashi and Y. Sumiki, This Bulletin, **21**, 73-4 (1957).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 4, p. 347, 1959]

Occurrence of Homoserine Dehydrogenase in Pea Seedlings

Sir:

Homoserine has been found in many plants[1]. Especially, this amino acid is formed in large quantities during the germination of the pea[2,3].

The author has undertaken to investigate the mechanism of homoserine formation in germinating pea seeds, and found that radioactive homoserine was formed remarkably, when pea seedlings were incubated in a medium containing C^{14}-labeled aspartic acid[3]. Moreover, the author detected in an extract of pea seedlings, an enzymatic activity catalyzing the reduction of TPN in the presence of homoserine[3]. The enzymatic activity was present in the 50~80 per cent acetone fraction. This result suggests the occurrence of homoserine dehydrogenase[4] in pea seedlings. In order to elucidate that this enzymatic activity is due to homoserine dehydrogenase, it is necessary to examine the reverse reaction of the dehydrogenation of homoserine (Aspartic β-semialdehyde+TPNH+H^+→Homoserine+TPN^+).

From such a point of view, the author tried to study the above reaction.

As shown in the figure, it was found that the oxidation of TPNH did not occur without the addition of aspartic β-semialdehyde, but occurred when aspartic β-semialdehyde was added at the point indicated by the arrow. When either TPNH or the enzyme was omitted from the reaction mixture, the changes of optical density at 340 mμ were not observed. These results show the occurrence of homoserine dehydrogenase in pea seedlings.

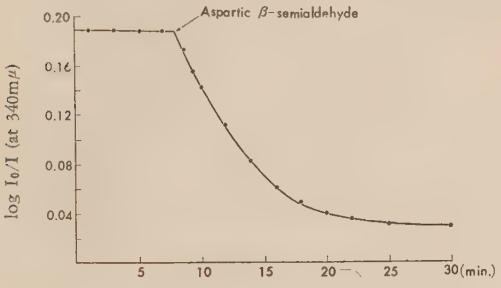

FIG. 1. Oxidation of TPNH in the Presence of Aspartic β-semialdehyde.

The complete system contained the following in a final volume of 3.0ml: 1.5ml of 0.2 M potassium phosphate buffer (pH 5.7); 0.09 μ mole of TPNH; approximately 10 μ moles of DL-aspartic β-semialdehyde; and 0.5 ml of the enzyme solution (50~80 per cent acetone fraction). DL-Aspartic β-semialdehyde was added at the point indicated by the arrow.

TPNH used in these experiments was prepared chemically according to Kaplan et al.[5] or enzymatically by the method of Evans and Nason[6]. DL-Aspartic β-semialdehyde was prepared from DL-allylglycine according to Black and Wright[7]. DL-Allylglycine was synthesized according to Goering et al.[8]

Light absorption at 340 mμ was measured with a Beckman model DU spectrophotometer (light path, 1 cm).

The author thanks Dr. S. Black for his kind gift of L-allylglycine.

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[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 4, p. 348~349, 1959]

The Preventive Effect of a S-Methyl Methionine Sulfonium Compound on Dietary Hypercholesteremia.

Sir:

Methionine, recently, has been marked as not only a lipotropic substance but also as an agent, which adjusts the level of serum cholesterol, and further, from the etiological point of view that hypercholesteremia is one of the representative symptoms of the disturbance of one-carbon fragment metabolism, resulting from the deficiency of active methyl compounds[1]. S-Methyl methionine sulfonium may participate in transmethylation, and thus, it shall be anticipated for having a preventing effect against hypercholesteremia. In this communication, we wish to call attention on this preventing effect of S-methyl methionine sulfonium iodide (MMSI) on experimental hypercholesteremia of rats, fed on a cholesterol enriched diet.

Twenty-three young and male albino rats of the Wistar strain, weighing 83.2±5.9 g (Group I-III, 14 animals) and 104.4±4.2 g (Group IV & V, 9 animals) were divided into 5 groups and fed on the test diet, consisting mainly of barley flour, defatted fish meal and refined lard, containing 1% of cholesterol, ad libitum, except for Group I.

MMSI was injected intraperitoneally to the test groups (Group III & V) at alevel of 20 mg per animal on alternate days throughout the experimental periods.

As control groups II and IV were injected with an equal volume of physiological saline or an equivalent iodine ion to MMSI, respectively. Group I was free from injection and cholesterol feeding.

MMSI was synthesized from DL-methionine and methyl iodide[2].

The determination of serum cholesterol was carried out as described by Bennie et al.[3], and that of cholesterol in the liver performed after the procedure of Gungbaum et al.[4]. Total lipid in the liver was estimated as an ether-soluble matter and the unsaponifiable matter was determined as the residual fraction of the lipid, by treatment with 30% potassium hydroxide.

The results are shown in the Table below.

The levels of total cholesterol in the sera of the animals, injected with MMSI (Groups III & V) are apparently lowerd as compared with that of the control groups, i.e., animals injected with saline (group II) and sodium iodide (Group IV). These data lead to some support of the view that MMSI might act as a preventing agent against dietary hypercholesteremia, and

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TABLE. CHOLESTEROL AND LIPID LEVELS IN THE SERA AND THE LIVERS

(mean±standard error of the mean)

Group No.	No. of rats	Weeks fed	Ch in diet (%)	Injected	Ch in serum		Ch in liver		Lipid in liver	
					Free (mg/dl)	Total (mg/dl)	Free (mg%)	Total (mg%)	Total (%)	unsap'd (%)
I	4	5	0	None	52± 7	195±25	233± 21	307± 52	9.08±2.29	0.62
II	5	5	1	NaCl	80±21	265±34	666±220	891±108	10.32±0.48	2.26
III	5	5	1	MMSI	82±29	200±48	716±151	1134±103	9.86±1.03	1.64
IV	4	3	1	NaI	59± 3	296±17	332± 12	709± 89	7.40±1.25	0.92
V	5	3	1	MMSI	50± 6	215±21	344± 83	884±112	8.64±0.78	0.88

Ch = Cholesterol.

this potentiality may be associated with methyl methionine sulfonium cation, but not with iodine anion.

The items measured, such as relative concentration of haemoglobin[5], protein concentration in the sera, some clinical test on the final-day urinary excretion, and microscopic observations of the liver section in fat-staining, did not reveal any remarkable alternation in the animals tested.

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Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 33, No. 5 (1959)

On the Catalyst Poisons from Sulfate Turpentine. Part I. Catalyst Poison in Dehydrogenation of Isoborneol. (p. *339*~*343*)

By Yoichi KABURAKI

(*Central Research Institute, Japan Monopoly Corporation*)

In the production of synthetic camphor from sulfate wood turpentine, a sulfur compound which inhibits the catalytic dehydrogenation reaction of isoborneol to camphor has been identified. A sulfone, m.p. 103.5~104°, obtained from contaminated isobornyl acetate by treating with oxidizing agent gives bornyl methyl sulfone, m.p. 61~61.5°, by potash fusion. This fact shows that the original sulfone is isobornyl methyl sulfone and the catalyst poison contained in isobornyl acetate is mainly isobornyl methyl sulfide, b.p. 100~100.5°/10 mm, d_4^{25} 0.9706, n_D^{25} 1.5058. The sulfide, highly toxic towards copper chromite catalyst, is transfered into isoborneol through the hydrolysis process of isobornyl acetate and inhibits the dehydrogenation reaction of isoborneol. Above two sulfones are nontoxic towards the catalyst and this interpretes the detoxication of contaminated isobornyl acetate by treating with chromic acid mixture.

On the Catalyst Poisons from Sulfate Turpentine. Part II. Formation and Autoxidation of the Catalyst Poison. (p. *343*~*347*)

By Yoichi KABURAKI

(*Central Research Institute, Japan Monopoly Corporation*)

The author has illustrated the formation and autoxidation process of isobornyl methyl sulfide, a poison of copper chromite catalyst, in the production of synthetic camphor from sulfate wood turpentine. Camphene prepared from the turpentine is contaminated with sulfur compounds, mainly with methyl disulfide, which, as methyl mercaptan, reacts with camphene in the presence of sulfuric acid in the process of acetylation of camphene to yield isobornyl methyl sulfide. The sulfide in isobornyl acetate is autoxidized in a long storage to yield isobornyl methyl sulfoxide, b.p. 123~125°/1mm, d_4^{25} 1.0670, n_D^{25} 1.5177, toxic towards the catalyst, and isobornyl methyl sulfone, nontoxic. This interpretes the facts that the toxicity of contaminated and long stored isobornyl acetate is found to be reduced and able to be removed by single distillation.

Inhibitory Effect of Ascorbic Acid on β-Amylase. Part VIII. On the Action Mechanism of Deinhibitors. (p. *347*~*350*)

By Masaharu ITO

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Annulling effects of some aminoacids and proteins on the inhibition of sweet potato β-amylase by ascorbic acid plus Cu^{++} have been studied.

The inhibition and also the autoxidation of ascorbic acid were annulled by the following aminoacids (except cysteine) arranged in order of decreasing effectiveness: histidine, cystine, tryptophane, tyrosine, glutamic acid, glycine and leucine. These results are depending on the stability constant of cupric chelate of the aminoacids. Proteins have also the same effect.

In addition to such a non-specific copper-binding capacity, proteins containing available SH group may have a specific annulling effect as the result of competition with SH group of enzyme for Cu^{+}. Thus, egg albumin and horse serum protein largely increased their deinhibiting power by heat denaturation.

Reaction of Furan Derivatives with Ammonia. Part III. On the Reaction of 2-Furyl Ethyl Ketone with Ammonia. (p. *351*~*353*)

By Hiroshi SUGISAWA and Kiyoshi ASO

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

Previous work has shown that 2-methyl-3-hydroxypyridine and 2-acetylpyrrole were obtained by reacting 2-acetylfuran with ammonia. This investigation was subject to a similar reaction in the hope that 2-ethyl-3-hydroxy-pyridine and 2-ethyl pyrryl ketone might be synthesized from 2-furyl ethyl ketone.

The authors have found it possible to obtain in good yield 2-ethyl-3-hydroxy-pyridine (II) and 2-ethyl pyrryl ketone (III) by reacting 2-furyl ethyl ketone (I) with ammonia at several conditions as shown in the table of present subject.

The reactants were heated in an autoclave at 180° for 20 hrs, the reaction mixture was treated with active carbon. After removal of solvent, III was distilled at $b.p._{12}$ 105~110°, and recrystallized from water, colorless needle, m.p. 52°. The residue was extracted with

2N-NaOH, and NaOH solution was saturated with CO_2, and then extracted again with ether for 48 hrs. II was recrystallized from chloroform-ether after sublimation, colorless prism, m.p. 134°. Its picrate was yellow needle, m.p. 173～174°.

Reaction of Furan Derivatives with Ammonia. Part IV. The Infrared Spectrum of β-Hydroxy-Pyridine Derivatives. (p. *353～358*)

By Hiroshi SUGISAWA and Kiyoshi ASO

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3-Hydroxy-pyridine (I), 2,3-dihydroxy-pyridine (II), 2-methyl-3-hydroxy-pyridine (III), 2-ethyl-3-hydroxy-pyridine (IV), 2-methyl-5-hydroxy-pyridine (V), 2-carboxyl-5-hydroxy-pyridine (VI) and 2-methyl-3,6-dihydroxy-pyridine (VII) having the characteristic structures respectively have been measured in solid state and solution, and the relation between structure and hydroxyl group was discussed.

I, III, IV and V did not show the absorption band at 3400 cm^{-1}, and have shown the hydroxyl group to be present in bonded form since they had two very broad bands centred at about 2450 cm^{-1} and 1800 cm^{-1}. Presumably OH—N intermolecular hydrogen bond exists and this accounts for the wide spread of absorption. II and VII having the tautomeric forms did not show intermolecular hydrogen bond.

α-Substituted pyridines had the absorption band at 990～1000 cm^{-1} and 1045～1050 cm^{-1}, and β-substituted pyridines at 1020～1030 cm^{-1} and near 1200 cm^{-1}. In addition, all the above compounds, α,β-disubstituted pyridines have shown two absorption bands described above. Moreover, the authors have pointed out that α-hydroxy-pyridines measured in this work had a characteristic band at 780 cm^{-1}, and β-hydroxy-pyridines at near 800 cm^{-1}.

Synthesis of Oligosaccharides by Growing Culture of *Leuconostoc mesenteroides*. Part II. Preparation of Kojibiose. (p. *359～362*)

By Kazuo SHIBASAKI and Kiyoshi ASO

(*Faculty of Agriculture, Tohoku University*)

A trisaccharide was isolated by the carbon column chromatography from a culture of *Leuconostoc mesenteroides*, which had been grown on a medium containing yeast extract, inorganic salts, sucrose and lactose. It is probably the same with the new "branched" trisaccharide which was characterised by Bailey, et al. as O-β-D-galactopyranosyl-(1,4)-O-[α-D-glucopyranosyl-(1→2)]-D-glucopyranose. On considering the structure, kojibiose and galactose will be obtained easily, if the 1,4-β-linkage (lactose) in the trisaccharide is cleaved by lactase.

The trisaccharide (10 g) and a lactase preparation from *Saccharomyces fragilis* (0.5 g) were added to 100 ml of phosphate buffer (pH 6.2) and the mixture were incubated at 35° for 30 hours. Five grames of kojibiose were isolated by carbon column chromatography. By acetylation two forms of crystals, each representing α- and β-octaacetate of kojibiose, respectively, were obtained. α-Form showed mp and mmp 166°, yield 1.7 g and β-form showed mp and mmp 117～118°, yield 3.5 g.

Also, by acetolysis of the trisaccharide (10 g) with acetic anhydride (50 ml), glacial acetic acid (32 ml) and concentrated sulphuric acid (6 ml) at room temperature for 7 days, 0.4 g of α- and 0.8 g of β-kojibiose octaacetates were obtained in crystalline form.

Studies on the N-Oxide and Amphoteric Surface Acitve Compounds. Part I. (p. *362～365*)

By Seishi SHIN'YA

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In this report the author stated the synthetic methods for preparation of several pure surface active compounds of quaternary, N-oxide and amphoteric (betain) type to investigate the relationship between chemical structure and antibacterial activity estimated by the standard cup method against *Staph. aureus* and *Esch. coli*. The compounds prepared were *n*-dodecylbenzyldimethylammoniumchloride (I), *p*-*n*-dodecylbenzyltrimethylammoniumchloride (II), *n*-dodecyldimethylamine-N-oxide (III), *p*-*n*-dodecylbenzyldimethylamine-N-oxide (IV), trimethyl-(*p*-*n*-dodecylphenyl)-methoxyammoniumchloride (V), N-*n*-dodecyl-N-dimethylglycin (VI), and N-*p*-*n*-dodecylbenzyl-N-dimethylglycin (VII).

n-Dodecylamine (b.p. 132～5°/3 mm) (A) reacted with 90% formic acid and 37% formalin to yield *n*-dodecyldimethylamine (B) (b.p. 110～2°/14 mm, n_D^{26} 1.4345, 95% purity, Y=81%). (B) 2.1g in C_6H_6 5 cc refluxed 5 hrs. with $C_6H_5CH_2Cl$ and evapd., gave 3.3 g crude (I) which did not solidify after extn. with petroleum ether and standing on P_2O_5 in vacuo over a week. (B) 2.1g in CH_3OH 10 cc treated with 2 g 35% H_2O_2 5 hrs. at 45°, evapd. in vacuo, and dried on P_2O_5 gave 2.4 g (III) m.p. 102° (AcOEt). (B) 2 g and 5 g $ClCH_2COOEt$ were refluxed 3 hrs. at 150～60°, and after saponification with alc. KOH and neutralization

of excess alkali by CO_2, crude (VI) was separated as a slightly colored crystalline from its aq. soln. at pH 4.2, m.p. 196° (EtOH) 2.3 g.

Lauric acid (C) (m.p. 45.1°, N.V. 276.0) was converted to its acid chloride (C) by thionylchloride and condensation of (D) with C_6H_6 in the presence of anhyd. $AlCl_3$ gave laurophenon, m.p. 44.5° (EtOH) (E) which was reduced to *n*-dodecylbenzene (F) (b.p. 145 ~8°/3 mm, n_D^{30} 1.4869) by Zn(Hg) and HCl in aq. AcOH. To the mixt. of (F) 28 g and 19 g $ClCH_2OCH_3$ (b.p. 57~60°) in 50 cc CS_2 was added dropwise 10 g anhyd. $SnCl_4$ below 0°, and the whole stirred 2.5 hrs. at 0°. The soln., poured into ice-water, gave 22 g crude chlormethylated prod. (G) which separated 9.4 g crystalline, m.p. 34.5°, after standing overnight at room temp. and the distillation of the filtrate gave more crystalline, m.p. 35°, 8.2 g. The total yield 54%. (G) 1.5 g and 40% aq. $(CH_3)_3N$ 1.0 g in 4.5 g EtOH was refluxed 3 hrs. and the soln. evapd., the residue crystd. from AcOEt gave (II), m.p. 191° (decompn.). (G) 4 g and alc. $(CH_3)_2NH$ (0.128 g/cc) 20 cc were heated in autoclave at 160~8° for 11 hrs., the soln. evapd. in vacuo, the residue taken up in 10 cc water, extd. with petroleum ether 20 cc, covered with ether and made alkaline by KOH. The ether soln., dried with solid KOH and evapd., gave 4.5 g crude *p*-*n*-dodecylbenzyldimethylamine (H), picrate m.p. 79.5°. Crude (H) 4.5 g, 35% H_2O_2 (d_4^{20}=1.112) in EtOH 25 cc stirred 6 hrs. at room temp., the solvent and excess H_2O_2 evapd. at 45° in vacuo. The residue, taken up in MeOH, was run into column packed with Amberlite IRC-50 (–COOH), eluted with MeOH. After removing top colored soln., 80 cc methanolic soln. collected, acidified with HCl, evapd. in vacuo. The residue, dried on P_2O_5, recrystd. from AcOEt gave (IV) m.p. 108°. (G) 1.5 g in *n*-C_3H_7OH 5 cc was refluxed 6 hrs. with $(CH_3)_3NO \cdot 2H_2O$ 0.6 g. After standg. at room temp. 0.2 g $(CH_3)_3N \cdot HCl$ separated. The filtrate, mixed with ether, precipitated more $(CH_3)_3N \cdot HCl$ and the filtrate evapd. in vacuo. The residue, recrystd. from AcOEt, gave (V) 0.6 g m.p. 169° (decompn.). The mother liquid, evapd. in vacuo, gave 1.1g semisolid m.p. 77°, which, recrystd. from EtOH gave *p*-*n*-dodecylbenzaldehyde m.p. 92°, 2,4-dinitrophenylhydrazone m.p. 161°. $ClCH_2COOH$ 1.9 g in 5 cc water was neutralized with $NaHCO_3$ 1.7 g and refluxed 2.5 hrs. with 25% aq. $(CH_3)_2NH$ 15 g. The aq. soln. evapd. on water bath to dryness, dried on H_2SO_4 overnight, recryst. from 10 cc EtOH gave 2.6 g crystalline. 0.6 g of this cryst. and (G) 1.4 g in iso PrOH 10 cc and water 2 cc refluxed 4.5 hrs., 20 cc ether was added to this soln. and the ppt. was filtered off, the filtrate evapd. The residue, extd. with 10 cc pet. ether, gave insoluble (VII) 0.3 g, m.p. 182°.

The results of antibacterial test were as follows. Compd., pH, the size of inhibition zone (mm) against *Staph. aureus* (S) and *Esch. coli* (E). (The conc. of the test soln. were 1.0% in all cases.)
(I) 6.2, 17.5 (S), 15.3 (E); (II) 5.4, 12.3 (S), 9.3 (E); (III) (a) 5.0, 14.3 (S), 10.8 (E) (b) 8.4, 10.0 (12.0) (S), 9.0 (E); (IV) 4.0, 10.8 (S), 11.0 (E); (V) 5.4, 10.8 (S), 11.0 (E); (VI) 4.0, 13.0 (S), 10.3 (E); (VII) 5.0, 11.8 (S), 9.8 (E).

The infrared spectra (Nujol) of (II), (IV), (V) and (VII) were also cited in this report.

Measurement of Protease Activities Using the Dinitrophenyl Protein as the Substrate. (p. *366~369*)

By Tadao HATA and Etsushiro DOI
(*The Institute for Food Science, Kyoto University*)

In the study of autolytic action of protein substances containing protease activity like meats, much trouble may be expected in finding the discrimination between the proteolytic products derived from the substrate proteins added and that from the enzyme preparation themselves used at that time.

Using dinitrophenyl substituted proteins as the substrate, seems to be more suitable for these investigations. The usual DNP-protein, however, can not be accepted for its less solubility.

So, the present authors happened to think of using the more soluble DNP-protein using dinitrobenzene sulphonic acid as the substituting reagent.

In the experiments with trypsin, papain and other proteolytic enzymes, the DNP-casein could be hydrolyzed easily like the ordinary casein. The proteolytic activities were measured at that time by the optical dentisity at 360 mμ of the filtrate, instead of by the optical density at 280 mμ when the usual protein are used.

The cange of absorption at 360 mμ with the time of hydrolysis, were parallel to the both changes of the absorption at 280 mμ and the amounts of free amino groups measured by Pope-Stevenes' method.

Moreover, as the blank became nearly zero, the measurements were easily carried out.

This method was applied to the measurement of proteolytic activity of rabbit kidney homogenate.

Studies of the Proteolytic Enzymes of Rabbit Muscle. (p. *369~374*)

By Tadao HATA and Etsushiro DOI
(*The Research Institute for Food Science, Kyoto University*)

Precise studies about the intracellular proteolytic enzymes in animal muscles seems to have important meanings not only in the studies of protein metabolism of living organisms but in the preservation and manufacturing of meat products.

Proteolytic activities of animal muscle, however, are so faint that the measurement of them can hardly be done due to the interferences by a large amount of inert proteins or peptides.

The present authors could measure the activities by eliminating these interference by the pretreatment to precipitate these inert substances.

In the fresh muscle extracts, thus, three kinds of proteolytic activities could be detected respectively in acidic, neutral and alkaline pH region. In the frozen stored muscle's extract, no activity was found at neutral pH region. In the acetone powder prepared from the fresh muscle, however, the activities were detected both at acidic and neutral pH region, but the latter one was so unstable that it was inactivated after a few days even in frozen storage. Although the proteolytic activity at pH 4 was unaffected by any reagent including reducing agent, EDTA and ferrous ion, the activity at alkaline pH was inhibited by phosphate ion and dinitrophenyl respectively.

The nature of these enzymes were discussed in comparison with the intracellular proteolytic enzymes of the other tissues.

Studies on the Chemical Constituents of Tea Leaves. Part X. Isolation of α-Spinasterol. (p. *374～375*)

By Yajiro SAKATO, Zenzaburo KUMAZAWA and Takashi MATSUMURA
(*Research Institute of Tea Industry, Kyoto; Laboratory of Agricultural Chemicals, Kyoto University*)

Theosterin which was previously isolated from the unsaponified part of ether extracts of tea was determined as α-spinasterol ($\Delta^{7,22}$-stigmastadien-3-ol) by the infra red-spectrometer.

Studies on the Components of the Green Leaves of *Ficus carica* L. Part III. Furocoumarins. (p. *376～379*)

By Shun-ichi FUKUSHI and Hiroshi TANAKA
(*Department of Agriculture, University of Tottori*)

The author distilled the green leaves of *Ficus carica* L. with steam, and from the distillate extracted crystalline components by petroleum benzine. The crystalline components were separated by the solubility of solvents, and from the components, insoluble by petroleum ether and soluble by benzene, psoralene, bergaptene and two crystals of m.p. 148° and 138° were isolated by column chromatography using an active alumina. Further, bergaptene was yield from the insoluble part of petroleum ether and benzene.

Psoralene was white needle crystals, m.p. 165°, $C_{11}H_6O_3$ and gave methyl derivatives of m.p. 162°. Bergaptene was fine white needle crystals, m.p. 190°, $C_{12}H_8O_4$ and did not give acetyl derivatives. Two white needle crystals of m.p. 148° and 138° were presumed to be the furocoumarin derivatives different from psoralene and bergaptene.

The ultraviolet spectra of psoralene exhibited the maxima at 247 mμ, 290 mμ and 326 mμ. Bergaptene at 250 mμ, 260 mμ, 268 mμ and 306 mμ. Two crystals of m.p. 148° and 138° at 247 mμ, 268 mμ and 296 mμ, respectively.

Studies on Microorganisms in "Shōyu Moromi." Part I. Main Lactic Acid Bacteria in "Shōyu Moromi." (p. *379～382*)

By Kazuhide YAMASATO and Hiroshi IIZUKA
(*Institute of Applied Microbiology, University of Tokyo*)

The NaCl content of Shōyu Moromi (soybean mash) is so high as about 18% and to considered to control the activities of microorganisms in it.

The authors searched for halotorelant bacteria really active in Shōyu Moromi using NaCl media.

From Shōyu Moromi at various stages of fermentation and ripening, acid producing tetrad cocci were isolated with overwhelming proportion to the total isolated bacteria on the media containing 7 and 15% of NaCl.

The cocci are catalase negative, microaerophilic, homofermentative lactic acid bacteria able to grow in 18% NaCl broth.

In the early stage of fermentation of Shōyu Moromi, the cocci increased in number; corresponding to this change the increase of total acid and lowering of pH were observed.

The cocci are considered to be one of the halotorelant bacterial group really active and main lactic acid bacteria in Shōyu Moromi.

The less or non halotorelant bacteria which had come from Koji-material were aerobic cocci, acid producing diplo cocci and rods.

Rods contain *B. megatherium*, *B. cereus*, *B. subtilis*,

B. pumilus, and Bacillus species which may belong to *B. circulance*.

While tetrad cocci were increasing in Shōyu Moromi, less or non halotorelant cocci were decreased in number and here remarkable change of microflora was observed.

Studies on Microorganisms in "Shōyu Moromi." Part II. *Pediococcus soyae* nov. sp. (p. *383*～*388*)

By Hiroshi IIZUKA and Kazuhide YAMASATO

(*Institute of Applied Microbiology, University of Tokyo*)

Surveying various characters of the tetrad cocci, the main lactic acid bacteria in Shōyu Moromi (soybean mash) reported in the previous paper, the authors recognized that the cocci all belong to one species of genus *Pediococcus*, though they produce *d*-lactic acid in contrast with other already known species which produce optically inactive lactic acid. The another distinctive character of them is their growth in NaCl broth. They are able to grow in media of high NaCl content i.e. 20% or more over.

They are not only merely halotorelant but halophilic; the character is genetic and inherited through serial transfer in the medium of low NaCl concentration.

The species is also recognized to be a new species and named *Pediococcus soyae* nov. sp..

Pediococcus soyae nov. sp.

Round cells, 0.6～0.9 micron in diameter, occurring singly, in pairs, or in tetrads.

Gram-positive, non spore-forming, and non motile.

Microaerophilic, catalase negative, nitrates not reduced to nitrites, gelatin not liquefied, and indole not formed.

Solid culture: Colonies in agar media are white, round or lens-shaped, 1～3 mm in diameter. Colonies on agar media are white, glistening, pulvinate or convex. In stab culture, filliform or pappilate growth, uniformly along the stab canal, no or very scant, if any, surface growth.

Liquid culture: Turbid visible after 24～48 hours, a firm sediment at the bottom of the tube. When old, liquid becomes clear.

Homofermentative lactic acid bacteria forming *d*-lactic acid.

Sugar fermentation: Acid from glucose, levulose, mannose, maltose, galactose and salicin. Very weak acid from lactose. Usually from xylose and α-methyl glucoside. No acid from sucrose, rhamnose, raffinose, inulin or dextrin. Arabinose, glycerol, and mannitole are fermented or not.

Growth temperature: Mesophilic, produce their maximum acidity between 25～30°, growth at 40° but not at 45°.

pH for growth: Opt. pH lies about 7.0, poor or no growth at 5.0.

Growth in NaCl broth: Halotorelant, able to grow in 20% NaCl broth. Halophilic, opt. NaCl concentration for growth is about 5%.

Main lactic acid bacteria in Shōyu Moromi (soybean mash).

Type strain has been deposited in Institute of Applied Microbiology, University of Tokyo, Japan.

Biochemical Studies of Rice Starch. Part IV. Sugar Phosphate, Monosaccharide and Oligosaccharide in the Seedrice at the Milky Stage. (p. *388*～*392*)

By Humio KURAZAWA, Ikuo IGAUE and Toshiro HAYAKAWA

(*Department of Agriculture, Niigata University*)

Sugar-phosphate, monosaccharide and oligosaccharide were found in the seedrice at the milky stage. Glucose-1-phosphate was observed by means of paper chromatography, and sucrose, glucose and fructose were recognized.

Biochemical Studies of Rice Starch. Part V. Phosphorylase in the Seedrice at the Milky Stage. (p. *393*～*398*)

By Humio KURASAWA, Ikuo IGAUE, Toshiro HAYAKAWA and Hiroshi OGAMI

(*Department of Agriculture, Niigata University*)

The seedrice at milky stage, after dipping in $Na_2S_2O_4$ solution (5 g/l) was crushed and pressed by a hand press. The juice was clarified by a refrigerated high speed centrifuge. The crude extract obtained was fractionated by ammonium sulfate. The precipitate by ammonium sulfate (25～35 g/dl) was found to contain phosphorylase. Authors presume that optimum pH of enzyme action was pH 6.6～6.7, and optimum temperature of its action was 36～37°.

Studies on the Decomposition of L-Ascorbic Acid. Part XI. Browning Reaction of L-Ascorbic Acid. (1) (p. *398*～*402*)

By Shintaro KAMIYA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University, Iwata*)

In this paper, it was observed that the browning reaction of L-ascorbic acid in orange juice was caused by dehydroascorbic acid, which easily formed some browing substances as reductones. On the other hand,

the mechanism of furfural formation from L-ascorbic acid was studied. L-Ascorbic acid, dehydroascorbic acid and 2-keto L-gulonic acid were decomposed by hydrogenchloride solution of 12% concentration and the amount of furfural formed was estimated by phloroglucin method. As the results, furfural was obtained with yield of 63.3%, 2.42% and 64.6% from the substrates respectively. Therefore, it was supposed that furfural would be formed via the following pathway, i.e.

L-ascorbic acid ⟶ 2-keto L-gulonic acid ⟶ pentose ⟶ (intermediate products) ⟶ furfural.

Studies on the Decomposition of L-Ascorbic Acid. Part XII. Browing Reaction of L-Ascorbic Acid. (2) (p. *402~406*)

By Shintaro KAMIYA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University, Iwata*)

In order to study the mechanism of browing reaction of L-ascorbic acid, dehydroascorbic acid was decomposed with sulfuric acid solution of 5% concentration. As the decomposition products, L-xylosone, reductone A (5-methyl 3,4-dihydroxytetrone), unknown substances (reductone B, reductone C), furan-2-carboxylic acid and a small quantity of furfural-like substance were detected by paperchromatographic and qualitative analyses. The mechanism of formation of these substances was supposed as follows:

Dehydroascorbic acid ⟶ 2,3-diketo L-gulonic acid ⟶ L-xylosone ⟶ 5-methyl 3,4-dihydroxytetrone (reductone A).
L-xylosone ⟶ furan-2-carboxylic acid, furfural-like substance.

From the above obtained results, it was considered that the browing reaction of L-ascorbic acid would be chiefly due to the formation of some reductones, which easily convert to browing substances.

Studies on the Classification of Baker's Yeast. Part I. Identification of Baker's Yeast of the World. (p. *406~411*)

By Tatsuro YAMAGUCHI and Akira FURUYA

(*Institute of Applied Microbiology, University of Tokyo*)

In order to know what is the baker's yeast and what is the differences among them in fundamental microbiological characters, 85 samples, including both commercial baker's yeasts and collection strains of them, were collected from U.S.A., Swiss, W. Germany, Czechoslovakia, Holland, England, Denmark, Itali, France, Sweden, Canada and Japan.

Identification of species were done according to the technique described by Lodder and Kreger-van Rij[1] and Wickerham[2], and all baker's yeast strains found to belong to *Saccharomyces cerevisiae* Hansen, though they had some minor differencies in such characters as pseudomycelium-forming tendency, cell size and shape, cell grouping tendency, ascospore formation and others.

Sacch. carlsbergensis, *Candida utilis*, *C. pseudotropicalis* were isolated respectively from collection strains which seemed not to be baker's yeast samples. *C. mycoderma*, *C. krusei* and few other anascosporogenic pellicle-forming yeasts were also isolated from baker's yeast samples as contaminants.

1) L. Lodder and N. J.W. Kreger-van Rij, The yeasts, a taxonomic study (1952).
2) L. J. Wickerham, *U. S. Dept. Agri. Tech. Bull.* No. 1029 (1951).

Studies on the Classification of Baker's Yeast. Part II. Macromorphological Characters and General Physiological Characters. (p. *412~416*)

By Tatsuro YAMAGUCHI and Akira FURUYA

(*Institute of Applied Microbiology, University of Tokyo*)

According to the previous paper all baker's yeast strains belonged to *S. cerevisiae*, but there seemed to have differences in microbiological characters among them. So other macromorphological and general physiological characters were scrutinized following the usual technique.

Especially noteworthy differences were observed on malt gelatin colony. Though majority formed Smooth type colony, three strains from Fleischmann Lab. and one strain from Central Research Dept. of Anheuser-Busch Co. formed remarkably Rough type colonies, and some other American, W. German, and Danish strains formed Intermediate type giant colonies. On malt ex.–yeast ex. agar in two weeks at 20°C similar characteristics were also observed.

Other differences which would serve as subsidiary characteristics in classifying baker's yeast were rapidity of utilization of ethanol in synthetic complete medium, growth in high sugar medium, and others.

Studies on the Classification of Baker's Yeast. Part III. Special Physiological Characters as Baker's Yeast. (p. *417~420*)

By Tatsuro YAMAGUCHI and Akira FURUYA

(*Institute of Applied Microbiology, University of Tokyo*)

As samples was containing both commercial baker's yeasts and stock cultures of them, comparison of special physiological characters as baker's yeast such as yield on molasses medium in shaking culture, yeast qualities like Meissl value, dough fermentation, flocculency were

examined, in order to excrude the degenerated collection strains and to know the relative qualities of these baker's yeast. More strict examination of practical characters of these strains, such as baking qualities and others expressed by incrementally grown yeasts, will be reported by Sato et al.

Among 79 baker's yeast studied 18 collection strains showed either one or more of the unfavorable characters such as very low Meissl value, no dough fermentation, and possesion of flocculency. In general some foreign commercial yeast strains showed excellent characters, whereas all of the Japanese baker's yeast strains stood in the middle. Most of collection strains were low in these characters but some still showed good results.

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
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Movement of Chlorine in the Rice Kernel. Part I. Inner Translocation of Chlorine in the Rice Kernel in its Storage. (p. *421~424*)

By Shoji KUBO and Chuichi TSUTSUMI

(*Food Research Institute, Ministry of Agriculture and Forestry*)

Monthly chemical analyses of rice kernel revealed that the majority of chlorine present in bran layer moved into endosperm, from April to July in the next year of harvest at an ordinary storage condition. During the season, the first state of equilibrium on the distribution of chlorine in the kernel seemed to give place to another stable state of equilibrium.

To this inner location of chlorine, a definite amount of water in the kernel was indispensable. Stored in the vessels in which relative humidity was 44%, no movement of chlorine occurred, even at room temperatures of spring and summer. Low temperature retarded the movement, but the transition was fully over before September, when the moisture content of the kernel was 16% or more.

The phenomena suggests the existance of certain different states of water in the rice kernel.

Movement of Chlorine in the Rice Kernel. Part II. Histochemical Observation of the Chlorine Distribution in the Rice Kernel. (p. *424~427*)

By Shoji KUBO and Kyoko FUJITA

(*Food Research Institute, Ministry of Agriculture and Forestry*)

Rice kernel was cut into a slice of 0.5 mm thickness, immersed in an alcoholic solution of silver nitrate for 7 minutes, and washed 2 to 3 times with alcohol containing nitric acid. The treated slice was exposed for 1.5 or 2 hours under a beam of ultraviolet rays, and then immersed in an alcoholic solution of hydroquinone to develop the figure of chlorine in the rice kernel. The result obtained showed a good agreement with that of chemical determination.

Using this histochemical method, it was confirmed that the chlorine at first present in pericarp and testa, aleuron layer and embryo moved and diffused into endosperm before the next summer of the harvest. It seemed that, once chlorine beginned to move inwards, less than 1 or 2 weeks was needed to come to the stable second equilibrium of distribution.

Histochemical dying of the rice embryo was found to be a particularly convenient method for the study of the movement of chlorine in the kernel.

Biochemical Studies of Rice Starch. Part VI. Enzyme which converts Iodine Color of Amylase from Blue to Reddish Violet in Ripening Seedrice. (1) (p. *428~432*)

By Humio KURASAWA, Ikuo IGAUE and Toshiro HAYAKAWA

(*Faculty of Agriculture, Niigata University*)

We have isolated enzyme Q-fraction from seedrice at the milky stage with the method of precipitation of blei acetate and salting out of ammoniumsulfate (18 g /100 ml).

When amylose is attacked by the enzyme Q-fraction, the blue color of the iodine reaction is converted to red without decoloration for 24 hours, and the formation of reducing sugar was 15% to the amylose. Optimum pH of the enzyme action is 6.6~6.8 and optimum temperature of its action is 33~34°.

The synthetic production from amylose is attacked by β-amylase, and saccharifying power in 67% is attained. In the case of amylopectin attacked by β-amylase, saccharifying power in 53% is attained; and in the case of amylose attacked by β-amylase, saccharifying power in 71% is attained.

Studies on the Utilization of Ion Exchange Process in Sugar Refining. Part VIII. The Relation between Decolorizing Effect and the Porosity of Strongly Basic Anion Exchange Resin by Using the Chloride Cycle.

By Susumu IWASHINA (p. *433~437*)

(*Research Institute of Meiji Sugar Mfg. Co. Ltd., Kawasaki, Japan*)

Previously it was reported by the authors that the structure of the R≡NX type anion exchange resins itself, especially porosity of it, had close relation to decolorizing effect, regeneration efficiency and color contamination when R≡NX type anion exchange resin in chloride form was used for the refining of sugar solutions.

In this report the weight of divinylbenzene (DVB) added as a so called crosslinking agent was changed

from 2% to 6% in molecular weight unit in the preparation of copolymer of styrene-divinylbenzene and the influence of the degree of porosity upon decolorizing effect, regeneration efficiency and color contamination was examined by preparing R≡NX type anion exchange resin of different porosity.

From the results of this experiments it was established that the leakage of SO_4 ion decreased proportionally with the ratio of DVB added in exchanging between Cl ion and SO_4 ion but decolorizing effect did not show necessarily the same tendency and that the mutual relation up to 15 cycles among decolorizing effect, regeneration efficiency and color contamination was investigated from color index, and the addition of DVB 3% gave the most balanced results.

Studies on the Utilization of Ion Exchange Process in Sugar Refining. Part IX. Long-Term Operating Characteristics of Strongly Basic Anion Exchange Resin by Using the Chloride Cycle. (p. *437~444*)

By Susumu IWASHINA

(*Research Institute of Meiji Sugar Mfg. Co. Ltd., Kawasaki, Japan*)

The purpose of the reports was to study on changes in several characteristics, as factors deciding the life of R≡NX type anion exchange resin, using Amberlite IRA-401 in chloride form, by continuous experiments as long as 400 cycles were carried out in the refining of sugar solutions.

Clarified sugar liquors applied with carbonation, active carbon and bone char methods as pretreatment was used and restorations as direct treatment for color contamination was carried out each every 10 cycles in two groups as (A) NaClO and HCl, (B) only HCl restorating procedures.

In the case of (A), they were used alternatively for comparative examination. The restoration level was used at 6 l/l-R as 0.5% solution in the case of NaClO and at 2 l/l-R as 3.3% solution in the case of HCl and the other conditions for penetration of liquor were based on the first report.

The results may be summarized as follows; The purifyning effect (A) and (B) were almost equal, lowering of basicity of the resin after 200 cycles was marked in (A) and differed clearly from (B), and ion exchange capacity of the resins after 400 cycles against control were (A) 48.7%, (B) 56.1% respectively. Contamination by inorganic substances in comparison with the control were (A)+16.78%, (B)−4.45% respectively and composition determined by spectroscopic analysis showed increase of Pb, Fe and Sn, and decrease of Mn and Ni both cases and no other marked differences were recognized.

Physical strength of the resin were decreased gradually both in (A) as well as (B). Especially in (A) superficial structure of the resin showed remarkable corrosion in observation by electron microscopy and the influence of duplicate effect of expansion and contraction during long usage together with the use of oxidizing reagent upon the physical strength lowering the resin matrix was recognized, thus giving suggestion to applying of restorations.

Moreover by infrared spectroscopic analysis with KCl disk method, color contaminated resins was observed to show the absorption at wave number of 1040, 1110, 1370 and 1600 cm^{-1} respectively and it was pointed out that the absorption other than 1040 cm^{-1} could be eliminated by restorating procedure but had a special feature to increase with the increase of the number of restorations.

It was estimated that absorption at wave number 1040 cm^{-1} suggested to alcohols or ethers, 1370 cm^{-1} together with 1600 cm^{-1} to –COOH linkage, and 1110 cm^{-1} to alcohols or aliphatic aldehydes or ketones or ethers linkage, showing some substances containing aboves.

Studies on the Chromatography of Starches. Part IV. On the Determination of the Amylose Contents of Various Starches by Paper Chromatographic Method. (p. *445~448*)

By Motoji TAKI

(*Faculty of Agriculture, Mie University*)

Filter paper strips were cleaned by washing with 15% KOH by the descending technique and air-dried after washing out in running water. The same treatment was repeated, using 35% $HClO_4$. Six lines, A, B, C, D, E and F, were marked with pencil 3, 6, 9, 11, 13 and 15 cm from the top edge of a strip (2×18 cm), respectively. Starch solution was placed between A and C as a wide zone and air-dried. The strip was developed with 35% $HClO_4$ by the descending technique until the solvent front reached to F. Since amylopectin fraction did not moved on the strip and amylose fraction descended to the part of the strip between E and F, immediately the strip was cut off along C and folded along D and then eluted with 35% $HClO_4$ by the descending technique. The amount of amylose in the eluate was estimated colorimetrically with iodine by referring to the calibration curve of amylose con-

centration vs. iodine color intensity which had been prepared by chromatographing the standard solutions of known amylose concentrations, using the same technique as described above. The amylose contents of various starches were determined by this method. Moreover, the amylopectin fractions which had been isolated from various starches by Schoch's method were chromatographed by the same technique. It was found that some of them contained a fraction which was stained blue with iodine. This fraction was considered as residual amylose in amylopectin fraction.

Studies on the Chromatography of Starches. Part V. On the New Method for the Paper Chromatography of Starches. (p. *448~452*)

By Motoji TAKI

(*Faculty of Agriculture, Mie University*)

The new method of the paper chromatography of starches was carried out for the determination of the amylose content of starches. Filter paper strips (2× 20 cm) were cleaned with 15% KOH and 40% $HClO_4$ solutions. Six lines, A, B, C, D, E and F, were marked with a pencil 3, 6, 9, 12, 15 and 18 cm from the top edge of a strip, respectively. Starch was dissolved in ice-chilled 40% $HClO_4$ solution. The strip was folded along line A and wetted to line C with 40% $HClO_4$ solution by the descending technique. Then, the strip was supported horizontally. 0.05 ml of the starch solution (5 mg/ml) was placed on the strip between line A and line B and allowed to diffuse beyond line B. The strip immediately was suspended in a descending chromatographic equipment containing 0.2 N I_2–KI solution and developed with the 40% $HClO_4$ solution which had been already introduced in the strip, in a refrigerator during a night. Amylose fraction retained as a blue stained zone between line B and line D. Amylopectin fraction descended to line F and was stained violet. The strip was irrigated with 40% $HClO_4$ solution until the amylopectin fraction was eluted from the strip. Then, the strip containing the amylose fraction was taken out from the chromatographic equipment and allowed to stand until the blue color almost disappeared. The amylose fraction was eluted with 40% $HClO_4$ solution. The amount of amylose in the eluate was determined colorimetrically with iodine.

Chromatographic Analysis of Glycerides. Part I. Paper Chromatography of Synthetic and Natural Glycerides as their Mercury Addition Compounds. (p. *452~456*)

By Yoshiyuki INOUE and Manjiro NODA

(*Biochemical Laboratory, College of Agriculture, Kyoto University, and Biochemical Laboratory, College of Agriculture, Kyoto Prefectural University, Kyoto*)

A new method for separating glycerides by means of reversed-phase paper chromatography is described. Twelve synthetic unsaturated mono-, di-, and triglycerides containing oleic or linoleic acid groups are well separated as their mercuric acetate addition compounds on paper impregnated with petroleum hydrocarbon (or tetralin). The developing solvent used is methanol—acetic acid—petroleum hydrocarbon (or methanol—acetic acid—tetralin), and the spots of the mercurated glycerides are detected by spraying with 0.2% ethanol solution of diphenylcarbazone. The method has also been applied to the separation of the component triglycerides in natural fats, which give characteristic chromatograms, respectively, depending upon their glyceride structure. The results obtained in these paper chromatographic analysis suggest that the separation of the triglycerides of natural fats on paper occurs principally affected by differing unsaturation of the individual triglyceride.

Studies on the Fine Structures of Fermenting Microorganisms by Ultra Thin Sectioning. Part I. On the Fine Structures of Conidia of *Aspergillus oryzae*, Spore of *Streptomyces griseus* and *Clostridium acetobutylicum*. (p. *457~461*)

By Shukuo KINOSHITA, Shiro ITAGAKI and Minoru FURUKAWA

(*Tokyo Research Laboratory, The Kyowa Fermentation Industry Co., Ltd.*)

Structures of several fermenting microorganisms were observed by ultra thin sectioning.

1. On the conidia of *Aspergillus oryzae* IAM S4–15.

(1) Conidia coats consisted of two membranes; outer membrane formed spine and inner one involved cytoplasm. The thickness of each membrane is about 50 to 100 mμ.

(2) Density of cytoplasm was not uniform. Low density area was presumed to be nuclear site. Also, several electron opaque intracellular granules were observed.

2. On the spore of *Streptomyces griseus* #1.

(1) The thickness of spore coat was about 25 to 50 mμ.

(2) Cytoplasm was usually uniform, but sometimes sponge-like structure was seen.

(3) The central area of cytoplasm was observed to be low density and it might perhaps be so-called nuclear site.

3. On the cell of *Clostridium acetobutylicum* KH 30–2.

(1) Cell wall and the basal granules of fragella were not able to observed.

(2) Cytoplasm consisted of very fine fibrous structure which was about 5 mμ in width.

(3) Continuous chain of large vacuoles were observed in cytoplasm. The chain usually consisted of 2 to 5 vacuoles.

(4) Thread-like structures which were about 20 to 30 mμ in width, were observed in vacuoles.

Occationally, these thread-like structures stretched out through the whole length of the chain of vacuoles.

Chemical Studies on Toxic Protein Ricin in Castor Bean. Part I. Research on the Separation of Ricin. (p. *461~464*)

By Masaru FUNATSU and Gunki FUNATSU

(*Biochemical Laboratory, Faculty of Agriculture, Kyushu University*)

Crystallin ricin and ricin Tb were prepared from two species of castor been seeds, *Ricinus sanginaus* L. and *Ricinus communis* L. It was discovered that crystalline ricin possessed one third of the proteolytic and one fourth of the hemagglutinating activity compared with those of ricin Tb, whereas both ricin possessed the same degree of toxicity. In addition, they were not regarded as a physiologically homogeneous protein. These facts suggest that there was a possibility of separating a protein responsible for the toxicity from the principles having proteolytic and hemagglutinating action.

In this connection, the purification of ricins, crystalline and Tb, is necessary for studying the physicochemical properties of toxic principles in ricin protein and the mechanism of the toxic action of ricin as well.

Chemical Studies on Toxic Protein Ricin in Castor Bean. Part II. Separation of Protease from Ricin by Continous Paper-electrophoresis. (p. *465~467*)

By Gunki FUNATSU

(*Biochemical Laboratory, Faculty of Agriculture, Kyushu University*)

Ricin Tb (*Ricinus communis* L.) and crystalline ricin (*Ricinus sanginaus* L.) were fractionated by continous paper-electrophoresis with borate buffer of pH 8.6 (μ =0.066).

Most of ricin moved to cathode, whereas proteolytic activity was present in the anode fractions. Toxicity was not found in the fraction possessing the heighest proteolytic activity, but in that possessing the lowest activity. This suggested that the toxicity was separated from the proteolytic activity.

Studies on Ascospores of Yeasts. Part I. On the Life Cycle of *Schizosaccharomyces* in Connection with "The Yeast Cell its Genetics and Cytology" by Carl C. Lindegren. (p. *468~469*)

By Kinshi SUMINOE and Jiro MIURA

(*Department of Agricultural Chemistry, Tokyo Agricultural University*)

In the book mentioned above, the author mentions that "this organisms has long cylindrical cells which become asci (spore sack) usually containing eight haploid spores each. The spores often fuse in pairs on germination to produce a diploid cell."

However upon tracing the life cycle of *Schizosaccharomyces octosporus*, we ascertain that in case of the said yeast, no copulation of ascospores occur, but vegetative cells copulate in pairs prior to sporulation. Therefore in *Schizosaccharomyces octosporus*, vegetative cells and ascospores must be both haploid, and diplophase is seen only in zygotes preceeding to sporulation.

Studies on Pungent Principles of Japanese Capsicum. Part III. Determination of the Pungent Principles (I). (p. *470~474*)

By Sadayoshi KOSUGE and Yukio INAGAKI

(*Faculty of Agriculture, Gifu University, Gifu*)

Capsaicin, which is a mixture of two pungent principles, is extracted with ethyl ether and, after evaporation of solvent, is taken up in 50 cc of CCl_4. The capsaicin in 5 cc of CCl_4, after being washed with dil-acetic acid, is taken up in 25 cc or 50 cc of 0.5N-NaOH solution.

A mixture (1:4) of Folin-Ciocalteu's reagent and 1M-NaH_2PO_4 is used as the color reagent. Each 5 cc of the alkaline solution to be analysed and 5 cc of the color reagent in a test tube (1.7 × 17 cm) are heated in boiling water for 7 minutes and, after being cooled, the absorbancy (E) of the obtained blue solution is measured at 750 mμ.

The capsaicin content (γ/5 cc) in the alkaline solution is calculated from the calibration curve, γ=190.5E−2.29.

When 0.5N-NaOH is used five times as much as CCl_4, the capsaicin content in the sample analysed is found by multiplying it in the alkaline solution by 57

(=1.14×50); When the times, the multiplier is 115 (=1.15×100).

No difference is found in pungency and above-mentioned absorbancy of pungent principles I and II. Accordingly, capsaicin, a mixture of principle I and II, has only to be analysed for acrid condiment.

Vanillin is found usuable, when submitted to the same procedures, as the standard for the determination of capsaicin. The relationship between capsaicin and vanillin is as follows;

Capsaicin (γ)=Vanillin (γ)×2.13

Proteolytic Enzymes of Butyl Bacteria. Part III. The Effects of Several Compounds on the Proteinase Activity of Acetone-Butanol Bacteria. (p. *474～477*)

By Shinji DOI, Yasuyuki KANEKO and Fuji UCHINO

(*Department of Agricultural Chemistry, Faculty of Agriculture, Nagoya University*)

The effects of several compounds and metallic ions upon the proteinase activity of acetone-butanol bacteria, strain 314, were studied. The following results were obtained.

1. The activity was not affected by sulfhydryl compounds and sodium cyanide.

2. Sulfhydryl group reagents, *p*-chloromercuribenzoate, iodoacetate, and ferricyanide, had no effect on the activity.

3. Metallic ions as mercuric, cadmium, and ferric ions markedly inhibited the activity, and only cobaltous ion elevated the activity by about 20%.

4. Some chelating agents such as ethylenediaminetetraacetate, nitrilotriacetate, and $\alpha\cdot\alpha'$-dipyridyl inactivated the enzyme and the addition of cobalt reactivated the enzyme once inhibited.

The experimental results suggest that the enzyme is not thiol enzyme, and some metal ions (i.e. cobalt etc.) are essential for the activity.

Studies on Myrosinase. Part I. Trials of Separating Myrosulfatase and Thioglucosidase and the Method of their Activity Measurement. (p. *478～483*)

By Zenji NAGASHIMA and Masaaki UCHIYAMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University*)

The mustard oil glucoside is split into glucose, mustard oil and potassium bisulfate, by an enzyme called myrosinase or myrosin.

The presence of this enzyme has been known early, but comparatively little is about its nature and action. Neuberg suggested that myrosinase is a mixture of two enzymes, myrosulfatase and thioglucosidase, which act on the substrate independently.

Their partial separation was made by fractional precipitation with mercuric acetate or absorption on kaolin, as he stated. The authors, attempted the separation by following procedure:

(1) fractional precipitation with acetone, alcohol or ammonium sulfate;

(2) electrophoretical separation using paper or starch carrier;

(3) fractional absorption on kaolin, Ca-phosphate and precipitation with mercuric acetate.

But their separation did not succeed in spite of many experiments under various conditions.

Moreover, the activity ratio of the two enzymes was almost constant (approximately 1) during the above procedures. Both the results thus obtained and the facts to be described in the next papers lead the authors to conclude the assumption of Neuberg doubtful.

Further, this paper examined the method for enzyme activity measurement.

Studies on Myrosinase. Part II. The Purification of Myrosinase and its Some Properties. (p. *484～487*)

By Zenji NAGASHIMA and Masaaki UCHIYAMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University*)

The previous paper shows that in spite of various attempts myrosinase could not be separated into two components, myrosulfatase and thioglucosidase, as formerly accepted. This paper presents a study of purification and properties of the myrosinase.

Myrosinase was purified about 200-fold by the application of various fractionations.

With this purified preparation, studies were made on some properties of both enzymes which show optimum pH at 5.0～5.2 and optimum temperature at 45～50°C. To the influences of pH and heat treatment on the activity both enzymes also showed entirely similar behavior.

Further, the activity ratio of thioglucosidase and myrosulfatase held constant during the purification. The facts thus obtained and described in the previous paper also make the authors to presume that the myrosulfatase and thioglucosidase activity belongs to a single enzyme protein.

Studies on the Action of Rennet on Whey Proteins. (p. *487～492*)

By Umeo YOSHINO
(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The action of rennet on β-lactoglobulin was examined. An amount of rennet enough to coagulate milk in cheese making was used throughout this experiment.

The inhomogeneity was observed in the β-lactoglobulin peak of the electrophoretic patterns of rennet whey and the albumin fraction from rennet whey at the pH below 7.5, but was not observed at the pH above 8.1 and at pH 4.8. The β-lactoglobulin peaks of acid whey and albumin fraction from acid whey were homogeneous at the all pH ranges examined.

From the results of electrophoretic analyses and the determination of phosphorous content, the inhomogeneity of the β-lactoglobulin peak of rennet whey and the albumin fraction from it was supposed to be due to the hydrolysis product from casein with rennet. The concomitant substance was removed with α-lactalbumin in the β-lactoglobulin crystallization process and electrophoretically homogeneous β-lactoglobulin is crystallizable from rennet whey.

The inhomogeneity of the β-lactoglobulin peak was not observed in the electrophoretic pattern of albumin fraction from rennet whey of heated milk (above 80°C for 30 minutes). The hydrolysis product of casein with rennet was thought to be salted out with heat denatured globulins and casein.

No significant changes in viscosity, ultraviolet absorption spectrum, electrophoretic patterns, solubility in salt solutions and formol titration value were observed in rennet-treated crystalline β-lactoglobulin solution. The appearance of the sulfhydryl groups in β-lactoglobulin solution with rennet action was not observed either.

Studies on the Enzymes of Lactic Acid Bacteria. Part X. Existence of Hexose-monophosphate Shunt System in the Cells of *Lactobacillus plantarum* No. 11. (p. *493*～*497*)

By Kakuo KITAHARA and Sakuzo FUKUI
(*Institute of Applied Microbiology, University of Tokyo*)

Modes of anaerobic cleavage of glucose caused by lactic acid bacteria have been classified into two types, viz. homo- and hetero-lactic acid fermentations.

In this paper it has been presented that no significant difference could be pointed out between homo and hetero fermenters in the gluconate metabolisms. *Lactobacillus plantarum* No. 11, a typical homofermenter, was able to produce ethanol, acetate and carbon dioxide besides lactate from gluconate just like as the case of heterofermenter, *Leuconostoc mesenteroides* B 07.

In the cell-free preparation from the "gluconate grown cells" of *L. plantarum*, the authors demonstrated the existence of following dehydrogenases: TPN-linked glucose-6-phosphate dehydrogenase, TPN-linked 6-phosphogluconate dehydrogenase, TPN-linked alcohol dehydrogenase and DPN-linked D- and L-lactic dehydrogenases. These dehydrogenases which had been proposed by DeMoss et al. as the members of heterofermenting system were also confirmed in the cells of *Leuconostoc mesenteroides* B 07.

From these findings it is suggested that the "gluconate grown cells" of *Lactobacillus plantarum* would be able to metabolize glucose through heterolactic acid fermenting pathway. But, the actual evidence could have never been obtained even in the presence of activators of glucose oxidizing system such as FAD, FMN and Vitamin K_5.

Studies on the Enzymes of Lactic Acid Bacteria. Part XI. Purification and Identification of "Coracemiase." (p. *497*～*499*)

By Kakuo KITAHARA and Akira OBAYASHI
(*Institute of Applied Microbiology, University of Tokyo*)

Purification of coracemiase was carried out by using the crude preparation as starting material which is easily obtainable by dialysis from cell-free extract of *Lactobacillus plantarum*.

The resulted preparation was about seven times stronger than the starting material both in coracemiase activity and absorptive power toward ultra-violet spectrum of 260 mμ.

This preparation was determined to have ribose, organic phosphate and adenine as its chemical components in the ratio of 1:2:1, and was shown by comparative experiments that it is nothing but diphosphopyridine nucleotide (DPN).

Pure specimens of DPN but not triphosphopyridine nucleotide can replace coracemiase.

Turnover number of DPN in the racemization of lactic acid was determined to be extraordinarily small (0.15 per minute). The misunderstanding in the previous paper [this Journal, 28, 232 (1954)] probably might be caused by this specificity.

An expectation may be born, from this specificity, that *L. plantarum* must contain abundant amount of DPN, and, certainly, as much as 5 mg of the coenzyme can be obtainable from each 1g of dried cells of the organism.

Studies on Growth Inhibition of Hiochi-bacteria, Specific Saprophytes of Sake. Part III. Activity of Antibiotics produced by Aspergilli against Hiochi-bacteria and its Estimation Method. (p. *500~504*)

By Teruo SHIRO and Seiji NAKAMURA
(*Yamamura Sake-brewing Laboratory*)

For the growth of true-hiochi-bacteria (*Lactobacillus homohiochii* and *Lactobacillus heterohiochii*), mevalonic acid (hiochic acid) is an indispensable growth factor as Tamura[1] reported. But peptides and other unknown stimulating factors are required by all the strains of hiochi-bacteria for their growth.

In Japanese Sake, the mevalonic acid and other these growth stimulating substances are contained in various percentages. In order to estimate activities of antibiotics produced by Aspergilli against hiochi-bacteria the authors used synthesized medium in place of diluted Sake medium[2] appling the Tamura's basal medium[3] which contained mevalonic acid and polypeptone for true hiochi-bacteria, and only polypeptone for hiochilactobacilli. On the shaking culture of modified Mayer's medium[2] authors obtained 18 strains of *Asp. oryzae* which showed activity against both of true hiochi-bacteria H-1 and hiochilactobacilli H-34, and on the rice suspending powder medium, 31 strains showed activity against them.

But on Koji-making method[2] only 5 strains of them prevented the growth of both of hiochi-bacteria.

1) G. Tamura, *J. Gen. Appl. Microbiol.*, **2**, 431 (1956).
2) S. Nakamura et al., *J. Agr. Chem. Soc. Japan*, **32**, 445 (1958).
3) G. Tamura et al., *J. Agr. Chem. Soc. Japan*, **32**, 701 (1958).

(*continued from front cover*)

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